



# PRINCIPLES OF SOIL MICROBIOLOGY

BY

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SECOND EDITION

THOROUGHLY REVISED



LONDON

BAILLIÈRE, TINDALL & COX

8 BENBETTA STREET, COVENT GARDEN, W.C. 2

1931



*This book is dedicated to Professors*

M. W. BEIJERINCK (✕)

AND

S. WINOGRADSKY

*the investigators who have thrown the first light  
upon some of the most important soil proc-  
esses and whose contributions can  
well be considered first and fore-  
most in the science of Soil  
Microbiology*

## PREFACE TO THE FIRST EDITION

Although the biochemical processes in the soil as well as the nature of the microorganisms present there have received considerable attention from various points of view and although an extensive literature has accumulated, not only dealing with soil processes in general but even with certain specific activities of the organisms, our present knowledge of the soil microflora and microfauna and of the numerous transformations that they bring about has not advanced beyond a mere beginning of a systematic study. The isolation of numerous microorganisms from the soil, their identification and cultivation upon artificial media is very important but such data do not tell what rôle they play in the soil. A knowledge of the activities of certain organisms isolated from the soil is certainly necessary, but that is not a knowledge of the extent to which these processes take place in the soil itself. A book on soil microbiology should include a study of the occurrence of microorganisms in the soil, their activities and their rôle in soil processes. It is this last phase which has been studied least and where the information available is far from satisfactory in explaining what is taking place in the soil. This is due largely to the limitations of the subject which depends for its advance on botany, zoology, bacteriology, chemistry, including biological and physical, and especially upon the advance of our understanding of the physical and chemical conditions of the soil.

There are various kinds of audiences to which a book on soil microbiology may appeal. There is the scientific farmer who may search for a better understanding of the processes taking place in the soil, those processes which control the growth of his crops and indirectly influence the growth of his animals. There is the agronomist, who is interested in the fundamental reactions controlling soil fertility, by reason of the need of directing such processes towards a greater utilization of the nutrients added to the soil or stored away in the soil organic matter. There is the investigator, the soil chemist or the soil microbiologist, who, in attacking problems dealing with the occurrence of microorganisms in the soil, their activities, and especially with the relation of these activities to the physical and chemical soil conditions, seeks for specific or general information. These investigators may deal with organisms or

processes which could be better understood when correlated with the other soil organisms and the numerous other processes. An attempt has been made to compile a book which will be of service not only to the investigators in soil science, but also to workers in allied sciences, especially botany, plant physiology, plant pathology and bacteriology, as well as to the general student in agriculture.

This book is a collection of known facts concerning microorganisms found in the soil and their activities; it is a study of the literature dealing with the science in question; it is an interpretation of the facts already presented; it indicates the various lines of investigation and notes where further information is especially wanted. Soil microbiology is a science which is at the very base of our understanding of agricultural processes and the practice of agriculture; it comprises a number of sciences. The book may, therefore, be looked upon more as an introduction to further research rather than as an ordinary text-book; as of help to those working in the allied sciences, who are desirous of obtaining some information concerning the soil population and its activities.

If this volume will help to disclose to the reader some of the numerous interrelated processes in the soil, if it will present in a clearer light to the chemist, the physiologist, the botanist, the bacteriologist and the zoologist the nature of the many scientific and practical problems awaiting the investigator, if it contributes in a small measure toward making soil science an exact science, the author will feel that he has been amply rewarded.

The author is greatly indebted to his various colleagues for reading and criticizing the different chapters of the book and for many helpful suggestions generously offered, especially to Dr. J. Blom, Mr. A. Bonazzi, Dr. B. M. Bristol-Roach, Dr. R. Burri, Dr. N. Cobb, Dr. H. J. Conn, Mr. D. W. Cutler, Dr. E. B. Fred, Dr. W. M. Gibbs, Dr. I. C. Hall, Dr. A. T. Henrici, Prof. D. R. Hoagland, Dr. L. R. Jones, Dr. W. P. Kelley, Dr. C. A. Kofoid, Dr. L. T. Leonard, Dr. J. G. Lipman, Dr. O. Meyerhof, Dr. G. T. Moore, Dr. T. B. Osborne, Dr. M. C. Rayner, Dr. H. Sandon, Dr. R. L. Starkey, Dr. J. Steiner, Dr. Ch. Thom and Dr. A. T. Whiting, as well as to all those who have generously allowed the use and reproduction of the various illustrations in the text.

SELMAN A. WAKSMAN.

*August 25, 1926.*

*New Brunswick, N. J., U. S. A.*

## PREFACE TO THE SECOND EDITION

Within the brief period of four years, since the appearance of the First Edition of this book, the numerous contributions to our knowledge of the rapidly growing subject of microorganisms and their activities in the soil necessitate a number of changes in the new Edition. The book has been brought to date by the incorporation of the additional information. A number of chapters have been entirely rewritten, especially those dealing with the mycorrhiza fungi and the soil as a medium for plant and animal parasites. A number of new chapters have been added, dealing with the rôle of microorganisms in the decomposition of organic matter in green manures and stable manures, in the formation and decomposition of peat and forest soils, and with the relation between plant growth and the activities of microorganisms in soil. To avoid any considerable increase in the actual size of the book, a certain amount of condensation became necessary so as to balance the added material. This was accomplished by leaving out some of the text which did not bear directly upon the subject under consideration. Several chapters have been combined so as to avoid unnecessary duplication.

As in the previous Edition, the interdependence between the activities of microorganisms and the chemical transformations in the soil has been particularly emphasized. The added chapters tend to stress further the influence of the soil as a medium upon the nature and activities of the microorganisms in the soil.

The author availed himself of the criticisms which have been so freely given in the various reviews of the first edition of this book that have appeared in English and in other languages; also of the numerous suggestions offered by various colleagues through correspondence. Opportunity is hereby taken by the author to express his gratitude to all his friends and colleagues in the various fields of microbiology and soil science for the generous assistance thus rendered.

In view of the rapidly growing bibliography of the subject of microbiology and its relation to soil processes, it seemed more desirable to leave out the titles of the papers rather than to have to sacrifice some of the older or even some of the more recent references to the literature.

SELMAN A. WAKSMAN.

*March 1, 1931.  
New Brunswick, N. J.*



# A CLASSIFIED LIST OF BOOKS FOR REFERENCE IN SOIL MICROBIOLOGY

## CLASSIFICATION OF ORGANISMS

### *Bacteria*

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TREATISES IN GENERAL SCIENCES

*General Biology, Physiology and Physiological Chemistry*

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PART A

THE SOIL POPULATION. OCCURRENCE AND ABUNDANCE  
OF MICROORGANISMS IN THE SOIL

*" . . . le rôle des infiniments petits m'apparaissait infiniment grand . . . "*

—PASTEUR





## CHAPTER I

### METHODS OF DETERMINING THE ABUNDANCE OF DIFFERENT GROUPS OF MICROORGANISMS FOUND IN THE SOIL. SOIL BACTERIA

*The soil population.* The soil consists, in varying proportions, of inorganic particles at different stages of subdivision and of different chemical composition derived from rock material and of organic matter originating from the decomposition of plants and animals. These particles are surrounded by films of water, which usually form a colloidal solution with some of the inorganic and organic matter. The spaces between the particles are filled with air and water. This soil is not sterile but is inhabited by an extensive microscopic population, consisting of numerous organisms which vary in size, shape, physiological activities, and their rôle in soil processes.

The nature and extent of the soil population depend upon the soil and the environmental conditions, including available food supply, proper aeration, adequate temperature, favorable moisture and reaction. Differences in the composition of the soil and in the nature of the environment will bring about marked differences in the kind and numbers of the microorganisms of the soil. A soil possessing certain physical properties, a definite chemical composition and a certain set of environmental conditions will also possess a well defined soil population belonging to the plant and animal kingdoms.

Any changes in the physical and chemical soil conditions and in the environment will be accompanied by quantitative and frequently even certain qualitative changes in the soil population.

Chart 1 gives a visual representation of the biological relationships of the various groups of microorganisms inhabiting the soil.

The animal world is represented in the soil by the protozoa, nematodes, rotifers, earthworms and various other worms as well as insects. The nematodes occur abundantly in all soils, but especially in greenhouse soils and certain infested field soils. Large numbers as well as numerous species of amoebae, ciliates and flagellates represent the protozoa in the soil.

The microscopic plant world is represented in the soil by the algae, fungi and bacteria, named in the order of their increasing importance of numbers and activities. Among the algae, the Cyanophyceae and Chlorophyceae are best represented. The soil fungi can be subdivided further into four groups:

1. Yeasts and yeast-like fungi, like the *Monilia* and *Oidia* (these two groups may, however, be classed with the true fungi).

2. Molds and other true fungi. Here we find the *Mucorineae* represented by the extensive genera *Rhizopus*, *Mucor*, *Zygorhynchus* and other *Phycomycetes*; various *Ascomycetes*, including the genus *Chaetomium* and other genera; *Hyphomycetes* represented by the *Mucedinaceae* (*Aspergillus*, *Penicillium*, *Sporotrichum*, *Botrytis*, *Trichoderma*, *Verticillium*, etc.), *Dematiaceae*, *Stilbaceae* and *Tuberculariaceae*.

3. *Actinomyces*. Ten to 50 per cent of the colonies developing from a soil on the common agar or gelatin plate belong to this important group of soil organisms. They are generally classified by bacteriologists with the bacteria; actually they belong to the fungi and are so far known to be represented in the soil by one extensive genus *Actinomyces*.

4. *Basidiomycetes*. These organisms comprising the higher or mushroom fungi are well represented in the soil; in forest soils they form a very extensive mycelium which probably predominates over that of other fungi. Many if not most of the mycorrhiza fungi belong to this group of organisms.

Bacteria predominate, in numbers and in the variety of activities, over all the other groups of microorganisms. This was the reason why the earlier microbiologists named the whole science of soil microbiology "soil bacteriology." It has long been recognized, however, that the soil population consists of various microorganisms other than bacteria, so that the more comprehensive term is fast coming into general use. Since the bacterial activities in the soil do not coincide with their taxonomic groupings, these organisms may be classified on the basis of their physiology for the sake of convenience in treatment. As a major division, the bacteria can be separated into two large groups:

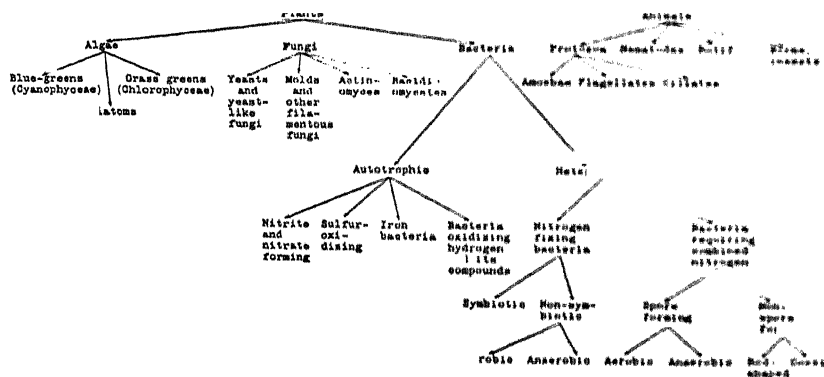


CHART I. The microflora and microfauna of the soil

(1) autotrophic, and (2) heterotrophic forms. Organisms that require for their nutrition substances which have been built up by other organisms are called heterotrophic. The heterotrophic bacteria consume, for their energy and for the building up of their protoplasm, the organic compounds of plant and animal bodies. Organisms like the green plants and certain bacteria that can thrive on purely inorganic substances and obtain their carbon from the carbon dioxide of the atmosphere are called autotrophic. The green plants derive their energy photosynthetically; however, the autotrophic bacteria derive their energy from the oxidation of purely inorganic substances, or chemosynthetically. The autotrophic group of bacteria is represented in the soil by smaller numbers and by much fewer species than the heterotrophic group, but it includes forms which are of greatest importance in the physiological processes in the soil, namely the organisms which oxidize ammonium salts to nitrites, nitrites to nitrates, sulfur and sulfur compounds to sulfates, and a few other less important groups.

The heterotrophic bacteria can further be subdivided on the basis of their nitrogen utilization: (1) Those bacteria that are able to fix atmospheric nitrogen in the presence of sufficient carbohydrates as sources of energy. This division is again only secondary in numbers, but its three representative groups play an important part in the soil economy, namely in the increase of the combined nitrogen of the soil. They are the symbiotic nitrogen-fixing, or nodule bacteria; the non-symbiotic aerobic nitrogen-fixing bacteria and the non-symbiotic anaerobic nitrogen-fixing bacteria. (2) Those bacteria which depend, for their metabolism, upon the nitrogen of the soil, in organic or inorganic forms. The heterotrophic non-nitrogen-fixing bacteria can be further subdivided, using as a basis either the need of free or combined oxygen or spore formation. The heterotrophic, non-nitrogen-fixing, aerobic bacteria are usually the organisms which are found on the plates, when an analysis of numbers of bacteria in the soil is made by the common agar or gelatin plate method.

It has also been variously claimed<sup>1</sup> that, in addition to the microscopic forms, ultramicroscopic organisms capable of passing through bacterial filters are present in the soil. Further studies could not prove the existence in the soil of such filterable organisms living in a free condition and capable of carrying out normal soil processes, independent of any

<sup>1</sup> Melin, E. *Ber. deut. Bot. Gesell.* 40: 21-25. 1922; Mische, H. *Biol. Centrbl.* 43: 1-15. 1923.

other soil organisms which are of importance in plant nutrition.<sup>2</sup> There is a possibility, however, that the soil harbors ultra-microscopic forms capable of living parasitically upon soil bacteria, as in the case of specific bacteriophages.<sup>3</sup> The importance of this phenomenon in soil processes has not yet been investigated.

*Proof of microbial activities in the soil.* The food requirements of the various groups of soil microorganisms are so distinctly different that no single artificial culture medium could be devised on which all of them could be studied. A large number of microbes, to which some of the most important soil forms belong, will grow only under very special conditions, such as selective media or selective environments. Various media and different methods have to be used for the study of the different groups. In some cases, special enrichment culture media favoring the development of particular organisms have to be devised, so that the growth of these will take place in preference to that of all the other organisms. Artificial conditions, which are distinctly different from those of the soil are thus created, and conclusions are based on the growth of the organisms under such conditions, which frequently do not hold true for a natural soil. To be able to grow the organisms in pure culture in the soil, the latter must be first sterilized. No method of sterilization has yet been devised which would not modify, in a fundamental manner, the chemical conditions of the soil. What will hold true for sterilized soil, then, may not hold true for unmodified soil. Again, the various organisms exist in the soil in large numbers, with a number of associative and antagonistic influences at work (both by living microorganisms and their products). Each organism has adapted itself to its environmental conditions and to the other organisms and may be, so to speak, in a condition of "unstable equilibrium." When this same organism is cultivated, in pure culture, upon a favorable medium, its activities are very likely to be different from those in the normal soil. Before we can conclude that a microorganism is active in the soil and that certain chemical transformations are produced by this organism under ordinary soil conditions, certain requirements must be satisfied. The following postulates, applied by Koch to pathogenic bacteria and modified by Conn<sup>4</sup> in their application to soils, should hold

<sup>2</sup> Rossi, G. *Soil Sci.* 12: 409-412. 1921; Barthel, C. and Bengtsson, N. *Meddel. 341, Centralanst. försöks. jordbr.* 1928. A recent note by Sherman and Safford (*Science*, 73: 448-449, 1931) indicates the probability that filterable microorganisms, capable of bringing about various decompositions are present in the soil.

<sup>3</sup> Dumas, J. *Compt. Rend. Soc. Biol.* 83: 1314. 1920.

<sup>4</sup> Conn, H. J. *Science. N.S.* 46: 252-255. 1917.

true for soil microorganisms: (1) The organism must be shown to present in the soil in an active form when the chemical transformation under investigation is taking place. (2) The organism must be shown to be present in larger numbers in such soil than in similar soil in which the chemical change is not taking place. (3) The organism must be isolated from the soil and studied in pure culture. (4) The same chemical change must be produced by the organism in experimentally inoculated soil, making the test, if possible, in unsterilized soil. (5) The organism must be found in the inoculated soil.

*Methods of study.* The methods generally employed for the study of soil bacteria can be divided into those of direct microscopic observation and cultural methods. The former have been suggested by Conn and further developed by Winogradsky. The latter have been used by the great majority of other soil microbiologists. Artificial culture media are employed, or at least artificial conditions are created. In many instances, therefore, no direct evidence is furnished as to what is actually taking place in the soil, under natural conditions. The results obtained under laboratory conditions often have to be interpreted as to their bearing upon actual field results.

*Direct microscopic method.* The method consists in preparing a suspension of soil in a dilute fixative solution, then spreading one or two drops of the suspension upon a clean slide, drying and staining with an acid dye. The fixative solution is prepared by dissolving 0.15 gm. of gelatin in 1 liter of distilled water with gentle heat; it is then distributed in test tubes, 5 cc. to each tube; these are plugged with cotton and sterilized. The staining solution is prepared by dissolving 1 gm. of erythrosin or rose-bengal in 100 cc. of 5 per cent aqueous solution of phenol containing 0.001 to 0.1 per cent  $\text{CaCl}_2$  to give a very faint precipitate of the calcium salt of the dye. The procedure of staining the soil organisms is carried out as follows: one-half gram of soil is placed in 4-5 cc. of the gelatin fixative solution. After thorough mixing, a large loopful of the suspension is placed upon a glass slide and spread out with a needle until it covers about 1 sq. cm. The smear is allowed to dry, preferably over a boiling water bath. A drop of the staining solution is then placed upon the smear, while the slide is still resting on the bath, and allowed to remain for 1 minute. The stain is then washed off quickly with water and examined under the microscope. A combination of lenses such as 1.9 mm. fluorite objective and 12.5 x compensating or planoscopic ocular will be found satisfactory.<sup>6</sup>

<sup>6</sup> Conn, H. J. N. Y. Agr. Exp. Sta. Tech. Bul. 64. 1918; Soil Sci. 26: 257-260. 1928; Jour. Bact. 17: 399-405. 1929. See also Whittles, C.L. Jour. Agr. Sci. 13: 18-48. 1923; 14: 346-369. 1924; Gray, P. H. H. and Thornton, H. G. Nature (London), 122: 400-401. 1928; Koffman, M. Centrbl. Bakt. II, 78: 337-352. 1929.

This method was modified by Winogradsky,<sup>6</sup> who found that the presence of large yellow grains of inorganic soil material hinders the proper examination of the field under the microscope. The soil samples are well mixed and powdered. One gram of the soil (on a dry basis) is then added to 4 cc. of distilled water and shaken vigorously for five minutes. After allowing to rest 30 seconds the suspension covering the large sedimented inorganic particles is poured off into a small tube of a hand centrifuge. Two 3-cc. portions of distilled water are then added to the residue, shaking each time one minute, allowing to rest 30 seconds and then pouring into the same tube of the centrifuge. Ten units of water are thus used for one unit of soil. After these three washings the first sediment suspended in distilled water settles immediately. During these manipulations, which require about 10 minutes, a second sediment is formed in the tube of the centrifuge. About half of the suspension is carefully taken out and placed in another centrifuge tube; on centrifuging, a third sediment is formed. Preparations are then made from each sediment and from the non-centrifuged and centrifuged suspensions. One drop of the various preparations is placed upon a slide covering just 1 sq. cm.; the preparations are dried in an oven and are rapidly covered with a very dilute agar solution. One per cent warm agar solution is best for the first two sediments and 0.1 per cent cold agar solution for the third sediment. For the suspensions, no fixative is necessary. When the agar is dried, several drops of absolute alcohol are used for fixing and the preparation is stained by means of a solution of an acid dye (erythrosine) in 5 per cent phenol solution. The bacterial cells are colored, but not the capsules and mucus; this is especially true of the compact colonies as those of *Nitrosomonads* and other soil forms which so readily over-color with basic dyes; the colloids are only faintly colored; the agar is readily decolorized by the process of washing with cold water. The dye is allowed to act 5 to 15 minutes in the cold or on slight warming, then washed a few seconds in water.

The preparations from the first sediment are usually free from bacteria, except in soils rich in organic matter, when some of the particles are not removed by

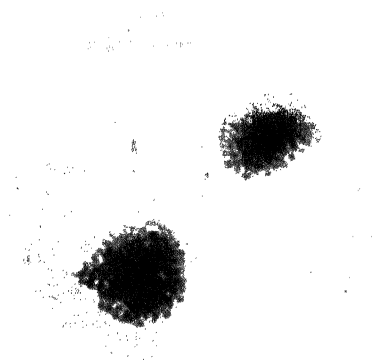
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<sup>6</sup> Winogradsky, S. Compt. Rend. Acad. Sci. 179: 367-371. 1924; Ann. Inst. Past. 39: 299-354. 1925.

## PLATE I

### MICROBIOLOGICAL POPULATION OF SOIL, AS SHOWN BY DIRECT MICROSCOPIC EXAMINATION

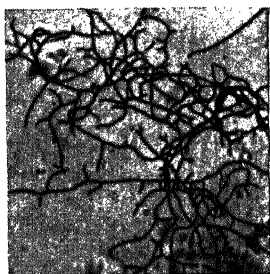
1. Colonies of *Azotobacter* type in soil, growing in the form of zooglea,  $\times 1000$  (from Cholodny).
2. Accumulation of large rod-shaped organisms around particles of organic matter,  $\times 1000$  (from Cholodny).
3. Actinomyces strands in soil (from Rossi).
4. The breaking up of Actinomyces filaments into spores, as shown by microscopic examination of soil,  $\times 1000$  (from Cholodny).
5. Fungus hyphae in soil,  $\times 200$ ; here and there are to be observed cells of bacteria (from Cholodny).
6. Soil amoeba feeding upon bacteria in soil (from Cholodny).



1



2



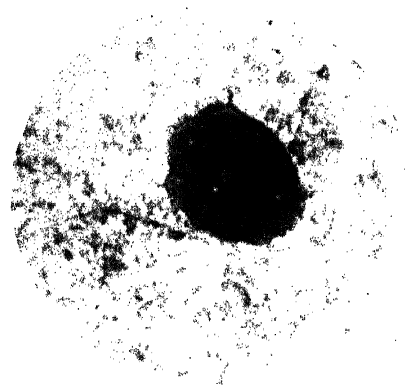
3



4



5



6





three washings. The second preparation shows on examination the same microbes, qualitatively and quantitatively, as the third sediment, where conditions for examination are most favorable. The fourth preparation made from the suspension is usually most instructive. The living cells only take the stain, while the spores stain only very faintly or not at all and can be seen only when present in large numbers. Protozoan cysts are recognized by their intense coloration and can easily be counted. The use of cyanosin for the staining of microorganisms by direct microscopic examination was recommended by Koffman.<sup>7</sup>

To avoid the phenomenon of adsorption of bacteria in soil, which interferes with their microscopic examination, it has been suggested<sup>8</sup> to treat the soil first with a normal solution of sodium chloride so as to destroy its colloidal complexes.

Winogradsky always used for comparison a control soil, which had no addition of fresh organic matter for a considerable period of time. A normal arable soil contains a native or autochthonous flora consisting of short bacteria with rounded ends and of cocci, 1 to 1.5 $\mu$  in diameter. Often larger forms, 1 to 3 $\mu$  in diameter, resembling *Azotobacter* are found. They group into rounded colonies consisting of about 100 cells in a compact mass with a common capsule, but occasionally with as few as a dozen individuals (Pl. I). The field between is completely devoid of microbes. The colonies are situated on the soil colloidal matter. This is the reason why the centrifuged suspension is practically free from colonies which are carried down by the flakes of organic matter. Spore-bearing bacilli, filamentous bacteria, spirals, mycelial filaments, actinomyces, and protozoan cysts are absent or are very rare. The presence of these indicates that the soil is in an active state of fermentation, due to recent addition of organic matter.

The fact that the soil represents a living system comprising numerous organisms having various functions and taking part in the numerous soil processes can best be observed by the microscopic examination of soil. The distribution of the soil population in response to environmental and nutritive conditions can also find an answer in these studies.

Cholodny<sup>9</sup> was fully justified in stating that the staining technique, as originally employed by Conn and Winogradsky, cannot give a complete picture of the soil population in its natural habitat, as a result of the shaking of the soil with water; this is especially true of the picture that one gets of the actinomyces in the soil, which is altogether distorted as a result of this treatment.

<sup>7</sup> Koffman, M. *Centrabl. Bakt.* II, 75: 28-45, 1928; 78: 337-352. 1929.

<sup>8</sup> Germanov, F. N. *Bull. 79, Nossov. Agr. Exp. Sta. (Russia).* 1927.

<sup>9</sup> Cholodny, N. *Arch. Mikrob.* 1: 620-652, 1930; see also Rossi, G. and G. Gesuè. *Ann. Tech. Agr. Roma.* 3: 196-248, 1930.

Cholodny proposed a new method, which he termed "soil plate method" or "surface growth plate method." A slit is made with a sharp knife in the soil which is to be examined. A clean cover slide is then inserted into this slit, parallel with the surface of the soil. The soil is then pressed gently to bring it in contact with the slide; surface of soil is covered, indicating with a convenient sign the position of the slide, which is now left in the soil for 1 to 3 weeks. The slide becomes covered with the soil solution and organic particles in and upon which various microorganisms immediately begin to develop. After 1-3 weeks the slide is carefully taken out of the ground by removing first the soil on one side of the slide; that side is then dried with a cloth to remove the soil particles. The slide is immediately dried in the air and placed in a proper container. In the laboratory, the preparation is first fixed by passing the slide over a flame, and washed gently in water to remove the coarse soil particles. The slide is then washed in distilled water, stained with phenol erythrosine (30 minutes at room temperature), according to Winogradsky's technique, washed thoroughly and dried.

The uneven distribution of the bacteria in the soil causing great irregularities and the difficulty of distinguishing bacterial cells from soil particles, especially in case of clay soils, and of separating living from dead bacteria make accurate counts impossible. The method can, therefore, not be used as yet for quantitative work, but is quite applicable for qualitative purposes, to show the types of microorganisms which exist in the soil in an active form. The microscopic method may be used for counting bacteria in culture media, especially in a liquid form.

*Organisms found in the soil by the direct microscopic method.* Conn demonstrated that the actual number of bacteria found in the soil, by the use of the microscope, is probably five to twenty times as great as that indicated by the culture plate method. This discrepancy is due to the fact that a large number of soil bacteria do not grow on the plates. By far the greatest number of microorganisms found in the soil, by the use of the microscope, consists of the minute non-spore-forming rods and cocci. The large spore-forming bacteria (as *Bac. megatherium* and *Bac. cereus*) have been found in normal soil only in the form of spores, which make up a very small proportion of the total bacterial flora of the soil. Filaments of actinomyces have also been found, but to a lesser extent than the spores of these organisms. The spore-forming bacteria became<sup>10</sup> active in the soil only when a great excess of easily decomposable organic matter has been added or when the moisture content of the soil is high. The minute non-spore-forming rods and cocci are considered to form the autochthonous microflora of the soil.

<sup>10</sup> Winogradsky, S. Compt. Rend. Acad. Sci. 178: 1236-39. 1924; Joffe, J. S., and Conn, H. J. N. Y. Agr. Exp. Sta. Bul. 97. 1923.

Conn<sup>11</sup> suggested to divide the bacteria observed under the microscope into six groups: large coccoid forms, small coccoid forms, long small rods, short small rods, large rods, and bacterial endospores. In soils with no added fresh organic material, the large rods and the spores are relatively scarce. According to Richter,<sup>12</sup> the microscopic examination of soil bacteria allows the differentiation of three distinct groups, namely (a) cocci and short rods, (b) typical large cells of *Azotobacter* and (c) bacillary forms. The first two groups are largely connected with the colloidal soil particles, in the form of zooglea, surrounded by slimy capsules; the third group is found mostly in the soil solution, but the representatives of this group occur frequently also in the form of clumps, especially on decomposing organic matter. A comparative study of the occurrence of these three groups at different depths of soil has given the following results:

TYPE OF SOIL	DEPTH	NUMBERS OF BACTERIA IN MILLIONS PER GRAM			YEAST CELLS	PIECES OF FUNGUS MYCELIUM
		Cocci	Azoto- bacter cells	Bacilli		
					<i>millions per gram</i>	<i>millions per gram</i>
Forest soil.....	Surface	1,379	156	1,212	1	47
	10 cm.	991	82	466	31	34
	20 cm.	281	188	169	—	7
Brown loam soil.....	Surface	870	188	376	84	5
	10 cm.	569	184	106	1	3
Sandy soil.....	Surface	519	155	192	79	3
	10 cm.	407	112	153	23	19
	20 cm.	269	51	139	8	3

The non-spore forming bacteria are thus found to be most abundant, the large rods or bacillary forms coming next, especially in soils rich in decomposing organic matter. Fungus mycelium is also abundant in such a soil.

Cholodny found that although forest soils contained an abundant flora of fungi, the latter organisms were not in excess quantitatively over the bacteria as is usually assumed. The fungi were present both as

<sup>11</sup> Conn, H. J. *Soil Sci.* 25: 263-272. 1928; *Tech. Bul.* 129, N. Y. Agr. Exp. Sta. 1927.

<sup>12</sup> Richter, A. A. and B. A. *Utechonie Zapiski, Saratov Univ.* 4: No. 1. 1925.

mycelium and in the form of spores, especially spores of Hyphomycetes. The hyphae of fungi were observed to serve as nutrients for the bacteria, various types of bacteria making extensive development at the expense of the fungus mycelium. Actinomyces were very abundant, although they could not be demonstrated to any extent by Winogradsky's technique, since on shaking the soil with water the mycelium is all broken up. The "streptococci" reported by Rossi and other observers are probably actinomyces spores. According to Chododny, the rod-shaped bacteria are in excess of the coccus forms in soil; among the former, one finds frequently spores and spore-forming types.

Algae developed only when the slide was buried in open places. Among the protozoa, the amoebae were abundant, especially where the bacteria were numerous. These organisms are feeding both on bacteria and on fungus mycelium. By burying the slide with a piece of paper, Chododny obtained a considerable development of fungi, in excess over the bacteria. The introduction of cellulose destroyed the "dynamic equilibrium" of the soil population, bringing about the development of a specific flora of fungi, followed by that of bacteria. Koffman found by direct microscopic examination that protozoa occur in soil in active stages. This is true of flagellates and rhizopodes. Ciliates were not observed in an active state in normal soil, but they appeared in large numbers after the soil had been kept for a few days in a moist condition.

The actual numbers of microorganisms as determined by the direct microscopic examination are usually found to vary considerably. Numbers ranging from 1,280,000,000 to 21,600,000,000 in 1 gram of soil have been reported,<sup>13</sup> with great variation between samples taken from different parts of the same soil area.

*Cultural methods for determining the numbers of microorganisms in the soil.* The cultural methods for the study of soil microorganisms are divided into methods for (a) quantitative determination of numbers of soil microorganisms, (b) qualitative studies, and (c) the measurement of microbiological activities, both in pure culture and in the soil. The earliest investigations in soil bacteriology were carried out<sup>14</sup> by the use of methods developed in medical bacteriology. The soil under investi-

<sup>13</sup> Vandeveld, A. J. J. and Verbelen, A. *Compt. Rend. Acad. Sci.* **190**: 977-979, 1930.

<sup>14</sup> Koch, R. *Mitt. K. Gesundheitsamt.* **1**: 34-36. 1881; Proskauer, B. *Ztschr. Hyg.* **11**: 22-24. 1892; Fränkel, C. *Ztschr. Hyg.* **2**: 521-582. 1887; Hiltner, L. and Störmer, K. *Arb. Biol. Abt. Land. u. Forstw., K. Gesundheitsamt.* **3**: 445-545. 1903.

gation was diluted with sterile soil, then plated out with gelatin and the numbers of bacteria determined after a certain incubation period. Later, sterile water was used for making the dilutions. In some cases small quantities of soil were weighed directly for the preparation of the plates. The method itself was imperfect and the results unrepresentative, and no relation was established between numbers and soil productivity. Hiltner and Störmer suggested the use of the dilution method, with the hope of doing away with the plate method, but here again the heterotrophic bacteria were determined by their growth on agar or gelatin media, while the autotrophic and nitrogen-fixing organisms were found not to be able to develop readily in high dilutions. Each of these two methods (plate and dilution) for determining the number of microorganisms in the soil has certain advantages and disadvantages.

The plate method consists in diluting the soil with sterile tap water, making a series of dilutions, so that 1 cc. of the final dilution, when plated out with nutrient agar or gelatin, will allow 40 to 200 colonies to develop on the plate. The dilution method consists of diluting the soil first with sterile water, as with the plate method, but transferring 1 cc. of several of the final dilutions into special sterile nutrient media adapted for the growth of particular groups of microorganisms. The number of cultures giving positive and negative growth allows an approximate determination of the number of specific organisms present in the particular soil.<sup>15</sup> The latter method is rather cumbersome, since it involves the preparation of a large number of media for the development of various physiological groups of organisms and the use of a number of containers for making the various dilutions, also, it involves great variability in the results.<sup>16</sup> The plate method is convenient, but its chief limitation is the fact that it allows the development of only the heterotrophic aerobic bacteria, of certain yeasts, molds and actinomyces. The dilution method will be found satisfactory for the study of practically all known soil microorganisms. The two methods may then be used each for its particular purpose, particularly in view of the fact that, for those microorganisms that develop on the common culture plate, the dilution method was not found by Hiltner and Störmer to give higher results than the plate method. The latter method should, therefore, be utilized for a general study of the numbers of microorganisms in the soil, keeping in mind its limitations, while the dilution method should be used for the determination of the abundance of special groups of microorganisms which do not develop on the plate.

<sup>15</sup> Löhnis, F. *Centrbl. Bakt.* II, 14: 1-9. 1905.

<sup>16</sup> Fischer, H. *Centrbl. Bakt.* II, 25: 457-459. 1910.

*Culture media.* With the introduction by Koch in 1881 of the gelatin plate for counting bacteria in general, an impetus was also given to the study of soil bacteria. But, unfortunately, Koch himself and practically all the bacteriologists following him for the next fifteen years were medical men interested particularly in the possible presence of pathogenic bacteria in the soil and their importance as carriers of infection. They quite properly, from their point of view, availed themselves of the methods of medical bacteriology. But even the excellent and stimulative researches of Koch and those following him<sup>17</sup> could not lay a proper foundation of soil microbiology, due primarily to the lack of proper methods.

The meat-extract-peptone agar or gelatin, found so valuable in pathogenic bacteriology, is entirely inappropriate for soil work, for various reasons, chief among which is the fact that the medium is not standard in composition and that it allows a rapid development of a few organisms which readily overgrow the plate and thus may prevent entirely the development of others. The distinct inferiority of bouillon agar or bouillon gelatin can be seen from the results of Engberding,<sup>18</sup> who found that a soil giving 99 colonies with Heyden agar, gave 39 with bouillon agar and only two with bouillon gelatin. But even the Heyden agar is not definite in composition, although it is often used for counting soil bacteria.

The media used for the determination of numbers of microorganisms in the soil (those that develop on the plate) should allow the development of the greatest possible number of organisms and should be standard in composition, so that every batch made up in the same laboratory or at any other laboratory will be like every other batch. This means that inorganic salts should be employed. If organic substances are necessary, they should be pure, stable, and standard if possible, as in the case of the carbon and nitrogen sources. Various sugars or organic acids serving as sources of carbon can be obtained in a standard form. Nitrogen substances should also be as standard as possible and added in as small amounts as possible. Agar in itself should not serve as a nutrient and should be, therefore, as pure as possible. To hold in check the development of certain rapidly growing organisms, which prevent the growth of the numerous but slow growing bacteria in the soil, the organic matter content of the media had to be reduced to a minimum.

The first important modifications in the composition of the medium

<sup>17</sup> Houston, A. C. Local Gov't. Board, Rept. 27: 251-296. 1898.

<sup>18</sup> Engberding, D. Centrbl. Bakt. II, 23: 569-642. 1909.

for a quantitative determination of soil bacteria were made by the introduction of the soil infusion agar,<sup>19</sup> and later by the elimination even of the soil extract,<sup>20</sup> using an agar, which contained only 0.05 gram of peptone per liter. The soil extract media do not, however, meet the qualification of being "standard in composition," since the soil infusion varies with the soil used for making the infusion. The synthetic medium was further modified<sup>21</sup> by the substitution of egg-albumin and casein for peptone. Among the other synthetic media suggested for the quantitative estimation of soil bacteria and actinomyces, sodium asparaginate agar,<sup>22</sup> asparaginate-mannitol agar,<sup>23</sup> and urea nitrate agar<sup>24</sup> should be mentioned. For the estimation of fungi, special acid media are required. For the protozoa, the dilution method still remains the most reliable, and nutrient agar or special liquid media can be employed for the development of the organisms in the final dilutions.

#### *Composition of media*

##### I. Fischer's soil extract agar:

Soil extract.....	1000 cc.
Agar.....	12 grams
K <sub>2</sub> HPO <sub>4</sub> .....	2 grams

The soil extract is prepared by heating soil for half an hour at 15 pounds pressure with an equal weight of a 0.1 per cent solution of Na<sub>2</sub>CO<sub>3</sub>.

##### II. Lipman and Brown's synthetic agar:

Distilled water.....	1000 cc.	Glucose.....	10 grams
Agar.....	20 grams	MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.20 gram
Peptone.....	0.05 gram	K <sub>2</sub> HPO <sub>4</sub> .....	0.50 gram

##### III. Albumin agar:

Distilled water.....	1000 cc.	MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.20 gram
Agar.....	15.00 grams	K <sub>2</sub> HPO <sub>4</sub> .....	0.50 gram
Powdered egg al- bumin.....	0.25 gram	Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> .....	trace
Glucose.....	1.00 gram		

The albumin is suspended in 5 to 10 cc. of water, then 1.0 cc. of 0.1N NaOH solution is added, so as to convert it into sodium albuminate; the albuminate is added only to the filtered medium.

<sup>19</sup> See Fischer, H. Landw. Jahrb. 38: 355-364. 1909.

<sup>20</sup> Lipman, J. G. and Brown, P. E. Centrbl. Bakt. II, 25: 447-454. 1910.

<sup>21</sup> Brown, P. E. Centrbl. Bakt. II, 38: 499-506. 1913; Iowa Agr. Exp. Sta., Res. Bul. 11, 396-407. 1913. Waksman, S. A. Soil Sci. 14: 283-298. 1922; Waksman, S. A. and Fred, E. B. Ibid. 14: 27. 1922.

<sup>22</sup> Conn, H. J. N. Y. Agr. Exp. Sta. Tech. Bul. 38. 1914.

<sup>23</sup> Thornton, H. G. Ann. Appl. Biol. 9: 241-274. 1922.

<sup>24</sup> Cook, R. C. Soil Sci. 1: 153-161. 1916.



IV. Casein agar. Same as medium III, only 1.0 gram of purified casein is used in place of the albumin. The casein is dissolved in 8 cc. of 0.1N NaOH.

V. Asparaginate agar:

Distilled water.....	1000 cc.	$\text{NH}_4\text{H}_2\text{PO}_4$ .....	1.5 grams
Agar.....	12.0 grams	$\text{CaCl}_2$ .....	0.1 gram
Sodium asparaginate..	1.0 gram	$\text{KCl}$ .....	0.1 gram
Glucose.....	1.0 gram	$\text{FeCl}_3$ .....	trace
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .....	0.2 gram		

10 cc. of 1.0N NaOH solution added per liter to bring the desired reaction.

VI. Asparagine-mannitol agar:

Distilled water.....	1000 cc.	$\text{NaCl}$ .....	0.1 gram
Agar.....	15 grams	$\text{FeCl}_3$ .....	0.002 gram
$\text{K}_2\text{HPO}_4$ .....	1.0 gram	$\text{KNO}_3$ .....	0.5 gram
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .....	0.2 gram	Asparagine.....	0.5 gram
$\text{CaCl}_2$ .....	0.1 gram	Mannitol.....	1.0 gram

The mannitol is added after the agar and other constituents have been dissolved and medium filtered. The reaction is adjusted to pH 7.4.

VII. Urea ammonium nitrate agar:

Distilled water.....	1000 cc.	Glucose.....	10.0 grams
Agar.....	15.0 grams	Urea.....	0.05 gram
$\text{K}_2\text{HPO}_4$ .....	0.5 gram	Ammonium nitrate.....	0.1 gram
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .....	0.2 gram	$\text{Fe}_2(\text{SO}_4)_3$ .....	trace

Reaction is about pH 7.0.

In addition to these media, soil extract and tap water gelatin recommended by Conn and used chiefly for qualitative purposes can also be mentioned:

VIII. Tap water gelatin.<sup>25</sup>

Tap water.....	1000 cc.
Gelatin (Gold Label).....	200 grams

These media are about equally favorable to the development of aerobic, heterotrophic bacteria, deriving their nitrogen from inorganic or organic compounds, and of actinomyces, but give somewhat different results for different soils, as shown by Conn. Egg-albumin agar and especially soil extract agar were found by others<sup>26</sup> to give higher and more uniform results than other media.

In preparing the media, the constituents including the agar or gelatin are dissolved in boiling water: after filtration through cotton, the more decomposable constituents are added, as in the case of urea, ammonium nitrate and glucose in the urea nitrate agar, albumin and casein solutions and the sodium asparaginate as well as the glucose, in the corresponding media, so as to avoid any possible effect of preliminary

<sup>25</sup> Medium is clarified by means of white of egg; reaction is adjusted to 0.5 per cent normal acid to phenolphthalein, which requires 20-30 cc. 1.0 N NaOH; when Bacto-gelatin is used, only 10 cc. of alkali is required and no clarification.

<sup>26</sup> Smith, N. R. and Worden, S. Jour. Agr. Res. 31: 501-517. 1925

heating on these substances. The reaction of the media is an important factor.<sup>27</sup> A slight acidity has usually been found to be the most favorable reaction for the development of the majority of soil bacteria. When determined by the hydrogen-ion concentration, colorimetrically or electrometrically, this reaction was found to be pH 6.5 (albumin and casein media) to pH 7.0 (asparaginate agar media).

The media are placed in 10-cc. portions in test tubes, or in 100-cc. to 200-cc. portions in Erlenmeyer flasks. These are plugged with cotton and sterilized in the autoclave, at 15 pounds pressure, for 15 to 20 min-

TABLE 1

*Influence of composition of media upon the numbers of bacteria as determined by the plate method<sup>22</sup>*

SOIL TYPE	BACTERIA, THOUSANDS PER GRAM DRY SOIL, AS DETERMINED WITH—				
	Soil-extract gelatin	Asparaginate agar	Lipman and Brown's agar	Fisher's agar	Albumin agar
Volusia silt loam.....	9,500	8,000	5,500	6,700	8,000
Volusia silt loam.....	12,500	7,500	5,300	5,000	7,000
Volusia silt loam.....	8,300	11,500	11,500	10,500	11,000
Volusia silt loam.....	10,000	12,000	8,800	12,000	11,000
Volusia silt loam.....	9,500	6,700	6,000	8,500	8,300
Dunkirk silty clay loam.....	9,000	14,500	14,000	11,800	15,000
Dunkirk silty clay loam.....	10,000	11,000	9,600	8,500	8,800
Ontario loam.....	21,000	9,000	13,000	15,000	14,000
Ontario loam.....	25,000	16,000	16,000	21,000	17,000
Volusia silt loam.....	5,000	5,500	5,500	5,400	5,500
Volusia silt loam.....	8,500	7,000	6,600	5,800	5,200
Volusia silt loam.....	10,500	6,000	9,800	9,000	6,300
Average.....	11,570	9,560	9,400	9,940	9,760

utes. A soil may contain 5 to 30 million bacteria (including actinomycetes) per gram and only 50,000 fungi or less. The final dilution used for the determination of bacteria would be 100,000 or 200,000. Some of the plates will have no fungi at all, while others may have one, two or more colonies. In this case the proper dilution for the determination of fungi would be 1000, but there will be so many bacteria on the plate that most of the fungi may actually fail to develop or make only a

<sup>27</sup> Clark, W. M. Jour. Inf. Dis., 17: 109. 1915. Clark, 1928 (p. xviii); Gillespie, L. J. Soil Sci. 9: 115-136. 1920.

scant growth. A medium should be used which allows only the development of fungi. Use is made of the fact that the majority of fungi can stand greater degrees of acidity than the bacteria and actinomycetes. For qualitative purposes and for pure culture study, any medium favorable for the development of fungi, such as raisin extract (60 grams of raisins heated at 60° to 75°C. in 1000 cc. of tap water, filtered), which is already acid in reaction, or a medium to which enough lactic acid is added to bring the reaction to pH 4.0. Because of the high acidity, 25 gm. of agar are required per liter.<sup>28</sup> For quantitative purposes, the following medium was found to give satisfactory results:

Glucose.....	10 grams	MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.5 gram
Peptone.....	5 grams	Distilled water.....	1000 cc.
KH <sub>2</sub> PO <sub>4</sub> .....	1 gram		

The ingredients are dissolved by boiling and enough normal acid (H<sub>2</sub>SO<sub>4</sub> or H<sub>3</sub>PO<sub>4</sub>) is added to bring the reaction to pH 3.6 to 3.8. This will require from 6 to 7 cc. of normal acid per liter of medium. Twenty-five to 30 grams of agar are added and dissolved by boiling; the medium is then filtered, tubed and sterilized. The final reaction of the medium should be pH 4.0.

For soils to which an excess of organic matter has recently been added, the dilution used for the determination of fungi is usually from one-hundredth to one-tenth of the dilution used for the determination of numbers of bacteria.

All the necessary glassware and water blanks should be prepared and sterilized before the soil samples are taken. Three or four pipettes and the same number of water blanks are required for every soil sample. Ninety-nine or 90 cc. portions of tap water, depending on the fact whether 1-cc. or 10-cc. pipettes are employed for making the dilutions, are placed in the flasks which are then plugged with cotton. The glassware (including the Petri dishes) is sterilized in the hot air sterilizer for two hours at 160°C., while the water blanks are sterilized in the autoclave, at 15 pounds pressure for 15 or 20 minutes. The sampling bottles may be sterilized either with the other glassware or with the water blanks.

*Sampling of soil.* When comparing different soils, samples should be taken from the same depth. The variability of a soil itself, even as to physical and chemical factors, is so great that it is not surprising that such a sensitive indicator as bacterial numbers and activities should vary considerably. To meet the factor of variation, the common practice has been to use composite samples, taken from a thorough mixture of several representative borings to the same depth, in various

<sup>28</sup> Waksman, S. A. Jour. Bact. 7: 339-341. 1922.

parts of the field. This may be sufficient, if the probable error involved in the determination is known. A study of the variability of bacterial numbers in the soil, as determined by the plate method, was found<sup>29</sup> to yield in one particular plot eight to twenty-five millions per gram. These results were based on fifty-one samples of soil taken from one-twentieth of an acre; the samples were taken at intervals of 5 by 8 feet. The numbers of bacteria of practically a whole series of plots variously treated and where definite bacteriological differences have been established were found to fall within that range of numbers. This would make any results obtained from a determination of numbers of bacteria based upon a single sample practically valueless. Before any definite conclusions can be drawn from a determination of bacterial activities in the soil, it is advisable to study the variability of the particular soil. This is done by taking a large number of samples from a particular field and working out the probable error of the particular variable which should not be greater than  $\text{E}_m = 2.5$  per cent.

For practical purposes, five composite soil samples each consisting of three to four borings taken from different parts of the field will give good results not only in the study of numbers of microorganisms but also in the study of specific physiological activities of soil bacteria.

In taking the samples, a small amount of soil ( $\frac{1}{4}$  inch) is scraped away from the surface by means of a spatula. The samples are taken by means of sampling tubes or augers, carefully cleaned previously, to a depth of 6 to  $6\frac{1}{2}$  inches, unless a study is made of the distribution of microorganisms at different depths. In that case a ditch may be made 3 feet long by 1 foot wide and, after scraping off the surface soil, at the desired depth, by means of a spatula, the samples are taken; small metallic sampling tubes (about 1 inch in diameter) made of iron or copper, with sharp ends may also be used for this purpose.

Care is taken not to contaminate the soil samples with other soil or by exposing them too long to the atmosphere. However, no absolute sterility in the process of sampling is required, since the numbers of microorganisms in the soil are very large in comparison with any possible contamination from a brief exposure. The samples are placed in sterile sampling bottles and brought to the laboratory as quickly as possible. A rapid change may<sup>30</sup> take place in the numbers of soil microorganisms when the soil is kept for a short time in the laboratory. Some investigators<sup>31</sup> found a strong decrease in numbers, on keeping the soil

<sup>29</sup> Waksman, S. A. *Soil Sci.* 14: 81-101. 1922.

<sup>30</sup> Fränkel, C. *Ztschr. Hyg.* 2: 521-582. 1887.

<sup>31</sup> Hiltner and Störmer, 1903 (p. 12).

sample for any length of time. An increase can be expected in soils, in which the activities of microorganisms have been kept in check, as in the case of dry soils moistened, soils treated with gaseous disinfectants, samples from low depths, frozen soils after they have thawed off. In the case of normal soils, a decrease may be expected; Hiltner and Störmer recorded a change in the numbers of bacteria from 7,519,000 to 2,087,000 in sixteen days and from 8,280,000 to 7,016,000 in six days.

The bacteriological analysis should, therefore, be made at once, or as soon as the soils can be conveniently brought into the laboratory.

*Treatment of soil samples and preparation of plates.* Before taking out portions of the samples for analysis, each sample is thoroughly mixed by means of a clean spatula. When many small stones are present, the sample is first sieved through a clean (sterile if possible) 2-mm. sieve. A definite portion of soil is transferred by any convenient method into an Erlenmeyer flask containing a definite quantity of sterile tap water, giving the first dilution. It is preferable to have the first dilution 1:10 or 1:20, i.e., to use ten or twenty times as much sterile water as soil taken. To give an initial dilution of 1:10, 10 gm. of soil are added to 100 cc. of water.<sup>32</sup> If the soil contains considerable organic matter, it may be advisable to triturate it first in a mortar with a little of the diluting liquid. The weight of the soil is either included in the total volume of the first dilution (10 grams of soil with sterile tap water to make 100 cc. volume) or not (10 grams of soil added to 100 cc. of water).

The mixture of soil and water is shaken for five minutes. This results in maximum numbers, shorter periods being insufficient to separate all the bacteria from the soil: longer periods of shaking have a destructive influence upon the bacterial numbers. The soil should not be allowed to be too long in contact with the water of dilution, otherwise an injurious effect will take place which is probably due to the plasmolytic action of the liquid. This leads to a rapid decrease in the number of microorganisms affecting the vegetative cells first. An injurious effect will occur only after 3-4 hours.<sup>33</sup>

In preparing the further dilutions, the contents of the flask from which the suspension is withdrawn should be kept in motion. The further dilutions can be prepared on the basis of 1:10 or to 1:100. It has been suggested<sup>34</sup> to limit the further dilutions also to 1:10, i.e., each higher dilution should be made by taking 10 cc. of the lower dilution and 90 cc. of the sterile diluting fluid. However, even by making higher dilutions such as 1:50 or 1:100 in each case, reliable results are obtained.

The amount of soil taken for preparing the first dilution, and the various subsequent dilutions are not of primary importance, if care is

<sup>32</sup> Wyant, Z. N. Soil Sci. 11: 295-303. 1921.

<sup>33</sup> Engberding, 1909 (p. 14).

<sup>34</sup> Noyes, H. A. and Voigt, E. Proc. Ind. Acad. Sci. 1916, 272-301.

exercised in the technic and in the calculations. By using for the first dilution 0.1204 gram of soil, Hiltner and Störmer found 9,500,000 bacteria in 1 gram of moist soil and, with 6.7841 grams of the same soil, 9,400,000 bacteria were found in 1 gram. In another instance, they found, by using 0.5862 and 21.1820 gram portions of soil, counts of 7,750,000 and 7,700,000 respectively per 1 gram of soil. The higher the dilution, the greater is the probable error of the results. The final dilution should be such as to allow between 40 and 200 colonies to develop on the plate, in the case of bacteria and actinomyces.<sup>35</sup> The number of fungus colonies allowed per plate on the special acid medium should be 20 to 100. This will necessitate that the soil should be diluted, in the counting of fungi, only to about 500 to 2000, so that the highest dilution is only 0.01 of that employed for the total bacterial numbers for the same soil. For soils very rich in organic matter or for highly acid soils, the ratio between the highest dilution for fungi and bacteria should be 1:50 or 1:10. Sterile tap water should be used for making the dilutions. Distilled water has a plasmolytic effect upon the bacteria. Salt solution or nutrient medium have no advantage over ordinary tap water.

The plates are prepared from 1 cc. of the final dilution. The number of plates to be used for each soil sample is important. The colony count varies greatly with the different plates, and results based on averages of two or three plates, without allowing for variability, makes them almost worthless. Hiltner and Störmer recognized this fact and used four and sometimes eight plates. The average error obtained was 8 to 10 per cent, in some cases even 15 per cent, the difference between the extreme variations being equivalent to one-third of the mean value. Variations equal to 33 per cent of the actual counts have been observed.<sup>36</sup> The variability may be somewhat decreased by very careful technic, but not reduced to a very small error.

*Incubation of plates and counting of organisms.* The plates are incubated at a constant temperature, preferably 25° to 27°C. for agar media, while gelatin media are incubated at 18°C. The plates for the determination of fungi are incubated for 48 to 72 hours; a shorter period of time is insufficient for the development of all the colonies, while a longer period favors rapid overgrowth of some fungi. The colonies are counted after 48 and again after 72 hours. The bacterial plates are incubated for seven to twelve days. A shorter period allows only an

<sup>35</sup> Breed, R. S., and Dotterer, W. D. N. Y. Agr. Exp. Sta. Tech. Bul. 53. 1916.

<sup>36</sup> Remy, Th. Centrbl. Bakt. II, 8: 657-662, 728-735. 1902.

insufficient development of the microorganisms.<sup>37</sup> When the plates are incubated only two or three days not all the organisms develop into visible colonies, especially the slow growing non-spore forming bacteria and actinomyces. Lower temperatures require longer incubation periods, so that, at room temperature, the plates should be incubated for fourteen days to give the maximum development of microorganisms, while, at 25°, seven days are sufficient. Higher temperatures, particularly above 30°C., have an injurious action upon the development of certain soil organisms; the medium in the plate also dries out rather rapidly.

At the end of the incubation period, all colonies on the plates are counted. Where only 40 to 200 colonies are allowed per plate, it is sufficient to make with a glass pencil two or three cross lines over the plate and count all the colonies with the naked eye. But if, for one reason or another, a lower dilution is used and the number of colonies exceeds 200 (a shorter incubation period may then have to be employed), the counting plate has to be utilized. In that case, of course, one has to depend on the manufacturer for the accuracy of divisions on the plate. A large enough number of sections should be counted so as to reduce the error involved in the determinations to a minimum. All the colonies, except the fungi, are counted and recorded as the total number of organisms developing on the plate, where no separate count of actinomyces is desired. Otherwise, after all the colonies have been counted, the actinomyces are determined separately. It is advisable to have recourse to the microscope for the examination of all the doubtful colonies, which are then marked off with a glass pencil. The plates may also be incubated a few days (4 to 7) longer and the actinomyces colonies counted. The number of actinomyces may then be subtracted from the "total number of organisms" to give the number of true bacteria.

The total number of organisms developing on the plate is recorded either on the basis of moist soil, stating thereby the moisture content of the soil, or on the basis of dry soil. The moisture is determined by drying 10 grams of soil at 100°C. to constant weight. If  $N$  is the total number of organisms developing on the plate from a given soil,  $a$  the average number for each plate,  $u$  the amount of dilution,  $x$  the moisture content of the soil, then,

$$N = \frac{a \cdot u \cdot 100}{100 - x}$$

*Mathematical interpretation of results.* The determination of numbers of microorganisms by the plate method involves a number of manipulations, each of which involves a certain error. The final error is a resultant of all these errors. The constant errors should be eliminated, but the occasional errors depend on the "Law of Chance" and these

<sup>37</sup> Conn, H. J. N. Y. Agr. Exp. Sta. Tech. Bul. 57. 1917.

tend to counterbalance one another rather than to fall in one direction. The results are calculated as follows:

If  $n$  plates are used for making a quantitative bacteriological count of a given soil,  $a$  the number of colonies developing on the plates, while  $z_n$  is the most probable value obtained from counting  $n$ -plates, or the arithmetic mean value, then we have

$$z_n = \frac{\sum a}{n}$$

In other words, the mean ( $z_n$ ) is found by dividing the sum of all the determinations by the number of determinations.

The following formulae are commonly used for calculating the average deviation and most probable error of the determination.

$$\text{Average deviation, } \sigma = \sqrt{\frac{\sum (Zn - a)^2}{n - 1}}$$

$$\text{Most probable error} = \pm 0.6745 \sqrt{\frac{\sum (Zn - a)^2}{n(n - 1)}}$$

$$\text{C.V.} = \frac{\text{the average deviation} \times 100}{\text{mean}}$$

$$\text{Em} = \frac{\text{most probable error} \times 100}{\text{mean}}$$

Approximately two-thirds of the determinations are expected to lie on both sides of the mean, within that deviation.

The probable error of the standard deviation is obtained from the formula  $\pm 0.6745 \frac{\sigma}{\sqrt{2n}}$ . The coefficient of variability (C.V.) is the percentage ratio of the standard deviation ( $\sigma$ ) from the mean. The probable error of the coefficient of variability =  $\pm 0.6745 \frac{\text{C.V.}}{\sqrt{2n}}$ . By the use of formulae for the calculations of the errors of observation, the error of the mean arithmetical value of the plate counts can be determined.<sup>23</sup>

By taking several representative soil samples and by using several plates, the error can be reduced to a minimum. A combination of five

<sup>23</sup> Davenport, C. B. Statistical methods. J. Wiley, New York. 1914.  
Barlow, P. Tables of squares, cubes, square roots, cube roots, reciprocals. Spon. London. 1914.



soil samples and ten plates, for each soil to be tested, will reduce the error for bacterial numbers to 1.8 per cent and for actinomycetes to 2.7 per cent.<sup>39</sup> The same is true of fungi; when a low dilution (1000) is combined with an acid medium, and 5 to 10 plates are used for each sample, the error may be reduced to 2 per cent.

It is of interest to note here that in order to determine accurately the average value of hygroscopic moisture in the soil, Floess<sup>40</sup> found that fifty soil samples, taken at definite distances in the field, are required. The probable error was calculated from the formula:

$$r = \frac{[v] \cdot 0.845}{\sqrt{n(n-1)}}$$

Where  $[v]$  is the sum of all individual variations from the mean and  $n$  the number of observations.

Under ideal conditions, the bacterial counts on parallel plates will vary in a manner similar to samples from a Poisson series.<sup>41</sup> The accuracy can thus be determined with precision and the mean count of a number of plates is a direct measure of the bacterial population capable of developing on the plate. Agreement with the theoretical distribution may be tested by means of the index of dispersion.

$$X^2 = \frac{1}{\bar{x}} \sum S (x - \bar{x})^2,$$

where  $\bar{x}$  is the mean and  $x$  any individual number of colonies counted on a plate;  $S$  stands for summation and  $X^2$  as the index of variability.

With a carefully improved technic, an accurate conformity with the theoretical distribution can be attained even with a mixed bacterial flora of the soil. Any significant departure from the theoretical distribution is a sign that the mean may be wholly unreliable.

*Comparison of plate and microscopic methods.* By comparing the results obtained by the microscopic and plate methods of determining the numbers of microorganisms in the soil, Conn<sup>42</sup> came to the conclusion that if an organism that can grow well on the plate is present in a soil, the plate count will be nearly as high or even higher than the micro-

<sup>39</sup> Waksman, S. A. *Soil Sci.* 14: 81-101. 1922.

<sup>40</sup> Floess, R. *Landw. Jahrb.* 42: 255-289. 1912.

<sup>41</sup> Fischer, R. A., Thornton, H. G. and Mackenzie, W. A. *Ann. Appl. Biol.* 9: 325-359. 1922.

<sup>42</sup> Conn, 1918 (p. 7); see also Kühlmorgen-Hill, G. *Centrbl. Bakt.* 11, 74: 497-519. 1928.

scopic count. In the case of large-sized bacteria, like *Bac. cereus*, the microscopic count was found to be higher than the plate count; in the case of small bacteria, like *Bact. fluorescens*, or the orange-liquefying type, which are easily overlooked under the microscope, the plate count is considerably higher. For the determination of the aerobic, heterotrophic, non-nitrogen fixing bacteria in the soil, which develop readily on the plate, the latter method is as satisfactory as the microscopic method. A number of bacteria, like the cellulose-decomposing forms, some urea bacteria and others, even among the aerobic heterotrophic organisms, are not capable of developing on the common plate, while some nitrogen-fixing bacteria may develop. The distinct difference between plate and microscopic counts of bacteria in normal soil is due not only to the organisms which are not capable of growing on common nutrient media, but also to the fact that a large number of cells, even of the organisms capable of growing on the common plate, do not develop into colonies. The actual number of bacteria in the soil is probably five to twenty and even more times as high as the counts obtained by the plate method.

*Numbers of bacteria in the soil.* In view of the fact that the methods used in the past for the determination of numbers of bacteria in the soil varied greatly and most of the media were not synthetic in composition, the results cannot be readily compared. However, certain fundamental facts have been established, which throw interesting light upon the nature of the soil population. By the use of the microscopic method Conn found, in one gram of soil, 140 to 390 millions of small rod-shaped bacteria less than 0.5 micron in diameter, 1,500,000 to 12,000,000 rods 0.5 to 0.8 micron in diameter and 500,000 to 5,000,000 spores per 1 gram of soil. Very few of the large rods (over 1 micron in diameter) were found in all the soil samples. In manured soils, the number of bacteria was found to reach 800,000,000 per gram, as determined microscopically. On the other hand, by the use of a nutrient agar plate, Adametz<sup>43</sup> found 320,000 to 500,000 bacteria per gram of sandy soil, and 360,000 to 600,000 in loamy soils. The numbers of bacteria in the soil obtained by the numerous investigators fall somewhere between these two sets of figures. These tremendous differences and variable results led Löhnis<sup>44</sup> to the conclusion that a determination of bacterial numbers in the soil is not of sufficient importance to attempt an interpretation of soil phenomena. However, when the results are considered critically and

<sup>43</sup> Adametz. Diss. Leipzig. 1886.

<sup>44</sup> Löhnis, 1910 (p. xiv), p. 513.

compared carefully, it is found that the information obtained from the study of numbers of microorganisms gives not only an interesting insight into the microbiological population of the soil, but also throws light on soil fertility, particularly when this information is combined with that obtained from the study of physiological activities of soil microorganisms.

In general the numbers of bacteria, as determined by the plate method, are found to range in normal soils well supplied with organic matter from a few hundred thousand to a hundred million cells per gram. These numbers vary with the soil type, soil treatment, season of year, depth, moisture content and various environmental conditions. No two soils, even from the same locality, can be compared on the basis of mere bacterial numbers without a detailed knowledge of the physical and chemical condition of soil, treatment, etc. In some soils, like very poor sandy soils, very acid peat soils and abnormal acid or alkaline soils, the number of bacteria may be much below 2,000,000 per gram. The sandy soils of the Pine barrens of New Jersey, for example, contain only 25,000 to 100,000 bacteria per gram, while certain acid peat and acid forest soils may be nearly free from bacteria capable of developing on the common agar or gelatin plate. Very heavily manured soils, such as greenhouse soils or market garden soils, particularly at the time of active decomposition of the organic matter, may contain over 200,000,000 bacteria per gram, even as shown by the plate method.<sup>45</sup>

*Bacterial numbers in manure.* Manure consists of (1) solid excreta, (2) straw, hay or peat litter, and (3) urine. Solid excreta are very rich in bacteria. The bacterial numbers in feces are very variable;<sup>46</sup> 1 gram of fresh human feces may contain 18,000,000,000 bacteria. Dry cow manure was shown<sup>47</sup> to contain, by weight, 9 to 20 per cent bacteria. One gram of fresh cow manure (17.65 per cent dry weight) should thus contain 18 to 40 billions bacteria, the larger number of which consists of dead cells. However, by the plate method, only 40 to 70 millions bacteria per gram of cattle manure and 100 to 150 millions per gram of horse manure were obtained. By the use of the gravimetric method, Lissauer<sup>48</sup> estimated that 4.26 to 15.67 per cent of the feces of man (on the average 9 per cent), 3.54 to 9.08 of dog, 0.41 to 1.31 of rabbit and

<sup>45</sup> More detailed information on the numbers of bacteria in peat soils is found in the Chapter on peat.

<sup>46</sup> Matzschita, T. Arch. Hyg. 41: 210-255. 1901.

<sup>47</sup> Stoklasa, J. Ztschr. landw. Versuch. Osterreich. 10: 440. 1907.

<sup>48</sup> Lissauer, M. Arch. Hyg. 58: 136-149. 1906.

14.73 to 18.75 per cent of the feces of cattle consisted of bacteria; the kind of food (vegetable or animal) influenced the numbers. Since 1 mgm. of dry bacteria contains 4,000,000,000 cells, 1 mgm. of cow feces would contain 63 to 80 millions of bacteria. By the plate method, manure of stall-fed cattle was found<sup>49</sup> to contain 1 to 120 million bacteria per gram, while that of cattle on pasture contained only 1 to 4 million. One billion bacteria were demonstrated<sup>50</sup> in 1 cc. of the intestinal contents of the ox.

In many of the earlier investigations too low numbers were recorded, due to faulty technic. In the more recent studies<sup>51</sup> 12,000,000,000 living microorganisms were found per 1 gram of compost of manure and straw; even these figures were too low, due to the fact that not all groups of organisms were counted. The feces of white rats was shown<sup>52</sup> to consist of 33 to 42 per cent bacteria by weight, although no attempt was made to differentiate between the living and dead cells.

The bacterial content of straw also varies greatly. Seventy-four thousand to 11,640,000 bacteria were found<sup>53</sup> per gram of straw and a decrease was observed as a result of drying and preservation under clean surroundings. The numbers of bacteria per gram of hay may vary between 10 and 400 millions per gram.<sup>54</sup> Figures varying from 3.6 to 600 millions of microorganisms per gram of straw were also reported. These organisms consist of non-spore formers (*Bact. herbicola*, *Bact. g ntheri*, *Bact. acidi lactici*, *Bact. fluorescens*), spore-formers, butyric acid bacteria, cocci, actinomyces (2 to 83 per cent of flora) and a few fungi.<sup>55</sup>

Urine is practically sterile, when it leaves the healthy body, but, on exposure, bacteria begin to multiply very rapidly, and soon an abundant flora consisting of bacteria and protozoa may appear.

The number of bacteria in stable manure will vary greatly, depending on its composition, the amount of solid excreta, and the degree of decomposition. The numbers will also depend of course upon the method used for their estimation, plate, dilution and microscopic methods giving

<sup>49</sup> Gruber, Th. Centrbl. Bakt. II, 22: 401-416. 1909.

<sup>50</sup> H ttermann, W. Diss. Bern. 1905; Koch's Jahresb. G rungs. 16: 402. 1905.

<sup>51</sup> L hnis, F. and Smith, J. H. F hl. landw. Ztg. 63: 153. 1914.

<sup>52</sup> Osborne, T. B. and Mendel, L. B. Jour. Biol. Chem. 18: 177. 1914.

<sup>53</sup> Hoffmann, F. Woch. Brau. p. 1153; Koch's Jahresber. G rungs. 7: 67-68. 1896. Esten, W. M. and Mason, C. J. Storrs (Conn.) Agr. Exp. Sta. Bul. 51. 1908.

<sup>54</sup> D ggeli, M. Naturw. Ztschr. Land. Forstw. 4: 473-492. 1906.

<sup>55</sup> K rsteiner, R. Centrbl. Bakt. II, 47: 1-191. 1916.

different results. Fresh manure is very rich in *Bact. coli*, which soon disappears in the composting of the manure. At first there is a multiplication of the bacteria, soon followed by a reduction in numbers, when the process of rotting of the manure is advanced. In manure fourteen years old and greatly reduced in volume, 12.5 millions of bacteria were found per gram (using manure extract-gelatin medium for counting), while manure preserved with superphosphate, gypsum and kainit contained 3.75 millions bacteria per gram.<sup>56</sup>

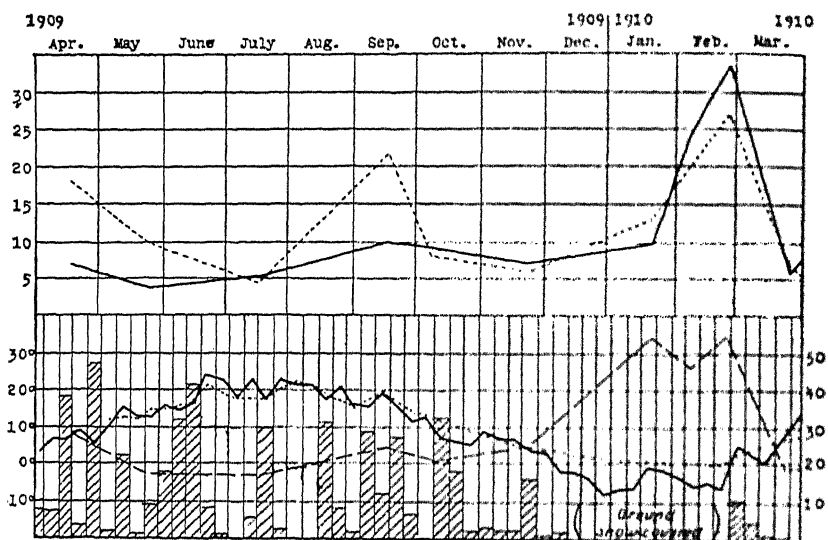


FIG. 1. Upper curves show millions of bacteria per gram of dry soil, throughout the year: ——— good soil, - - - - - poor soil; lower curves show: ——— soil moisture in per cent; ——— atmospheric temperature; - - - - - soil temperature; shaded columns represent rainfall per week, in millimeters (from Conn).

*Numbers of bacteria in the soil during different seasons of the year.* The season of the year cannot be considered as one single variable, so that its influence upon bacterial numbers and activities could be studied. The moisture content of the soil at the particular date of sampling, kind of crop, stage of growth of crop, previous soil treatment, and many other factors will influence the numbers and types of microorganisms found in the soil. Few bacteria may be found in the soil at the end of a long dry period. After the first rainfall, there is a rapid increase in

<sup>56</sup> Löhnis, F. and Kuntze, W. Centrbl. Bakt. II, 20: 676-687. 1908.

numbers, due to the fact that a continued dry period brings about a phenomenon similar to partial sterilization of the soil and with the first increase in moisture there is an increase of available organic and inorganic materials, leading to a rapid increase in bacterial numbers. Several investigators found larger bacterial numbers in the soil in summer than in winter, the maximum being reached in July and August. In the spring of the year, with the increase in soil temperature, there is a corresponding increase in bacterial numbers.<sup>57</sup> Engberding<sup>33</sup> suggested, however, that the water content of the soil is the most important factor bearing upon bacterial numbers: cultivation increases the number by increasing the water content; he was unable to demonstrate any influence of temperature upon the numbers of bacteria in mineral soils. This difference in results may be due to the difference in the composition of the soil. The numbers of bacteria (and protozoa) were even reported to fluctuate from day to day.<sup>58</sup> Well-marked seasonal changes in the soil population are superimposed on the daily variations in numbers. These changes are not directly influenced by temperature or rainfall, but show a similarity to the seasonal fluctuations recorded for many aquatic organisms.

Conn,<sup>59</sup> after a careful comparison of bacterial numbers in frozen and unfrozen soil, came to the conclusion that the number of bacteria in frozen soil is generally larger than in unfrozen soil, which is true not only of cropped soil, but also of sod and fallow land. This increase in bacterial numbers after freezing was believed not to be due to an increase in moisture content, even though in an unfrozen condition the bacterial numbers seemed to increase and decrease parallel to the moisture content of the soil. The increase in frozen soil seemed to be a result of actual multiplication of the bacteria, rather than of a mere rise of the organisms from lower depths brought about by mechanical forces alone. Conn, therefore, suggested that there are two groups of bacteria in the soil, "summer" and "winter" bacteria, which are active in the respective periods. These results were at first confirmed.<sup>60</sup> The theory was suggested that surface tension exerted by the soil particles

<sup>57</sup> Remy, 1902 (p. 21).

<sup>58</sup> Cutler, D. W., Crump, L. M. and Sandon, H. Phil. Trans. Roy. Soc. London, B, 211: 317-350. 1922.

<sup>59</sup> Conn, H. J. Centrbl. Bakt. II, 28: 422-434. 1910; 32: 70-97. 1911; 42: 510-519. 1914; N. Y. Agr. Exp. Sta. Tech. Bul. 35. 1914.

<sup>60</sup> Brown, P. E. and Smith, R. E. Iowa Agr. Exp. Sta., Res. Bul. 8: 281-321. 1912.

on the films of water, as well as the presence of salts in the water and the concentration of salts, which may occur when the main body of water begins to freeze, all cause the hygroscopic water in soils to remain uncongealed, and consequently bacteria may live in it and multiply to a comparatively large extent. Other results<sup>61</sup> also tended to indicate that low temperatures may greatly increase the numbers of bacteria. To explain these phenomena, the mechanical transportation of the bacteria by the moisture coming up from below during heavy frost was suggested.<sup>62</sup>

It was later found, <sup>63</sup> however, that a slightly frozen condition of the soil allowed bacterial development, but severe frosts produced a checking action, the decrease being parallel with the depression of temperature. No change in crop and plant remains took place in Canada during the winter since the temperature of the soil goes down too low. The occurrence of two maximum counts of bacteria observed<sup>64</sup> in Iowa soils during the year, on February 12 and June 19, with intervening minimum counts, were used to prove the theory that temperatures much below zero are necessary before the hygroscopic moisture freezes and, until that occurred, a development of bacteria might be expected. It may also be suggested that a slight freezing of the soil, in modifying the colloidal condition of the soil may have the same stimulating action as air drying, treating with disinfectants, etc., in other words, shifting the soil equilibrium, so that a more rapid multiplication of the bacteria may take place.

A more recent careful study of the influence of freezing upon soil bacteria demonstrated that the increase recorded previously is not due to an actual multiplication of the bacteria but rather to a breaking up of the clumps of bacteria in the soil resulting in a larger number of colonies developing on the plate<sup>65</sup>. The moisture content and rate of thaw are important factors, in determining the extent of the breaking up process. The work of Lochhead<sup>66</sup> also tends to refute the idea that there are in the soil two groups of bacteria, winter and summer forms. Although the numbers of bacteria in frozen soil are high, there is no

<sup>61</sup> Weber, G. G. A. Diss. Jena, 88. 1912. (Centrbl. Bakt., II, 37: 113. 1912.)

<sup>62</sup> Harder, E. G. Bot. Gaz. 61: 363. 1916.

<sup>63</sup> Vanderleek, J. Trans. Roy. Soc. Canada (Ser. III, Sec. IV), 11: 15. 1918; 12: 1. 1918.

<sup>64</sup> Brown, P. E. and Halversen, W. V. Iowa Agr. Exp. Sta., Res. Bul. 56, 1919.

<sup>65</sup> Vass, A. F. Cornell Univ. Agr. Exp. Sta. Memoir 27. 1919.

<sup>66</sup> Lochhead, A. G. Trans. Roy. Soc. Canada. 18: 75-96. 1924; Soil Sci. 21: 225-232. 1926.

phenomenal increase, as a result of freezing, while thawing of the soil brought about a great increase in numbers. A typical winter flora is absent and the bacteria are to be regarded as cold-enduring rather than psychrophilic in the true sense. There was also no indication of a rise of organisms from unfrozen levels to the frozen surface layers.

*Distribution of bacteria at various soil depths.* In humid soil, most of the bacteria are concentrated in the upper 2 feet of soil and the highest numbers are found just 1 to 3 inches below the surface. The germicidal effect of the rays of the sun and the rapid evaporation of the moisture tend to diminish the numbers right at the surface. Much fewer bacteria are present in the subsoil; in sandy soils, due to better aeration, the numbers do not diminish as rapidly. Since most of the earlier work was limited to the use of the plate method, the results are of necessity too low.

Proskauer<sup>67</sup> was the first to point out the fact that the numbers of bacteria in humid soils decrease with depth; at a depth of about one meter the soil was found to be almost free from bacteria. Fränkel<sup>68</sup> observed a decrease of bacterial numbers with depth of soil, from 90,000 to 300,000 at the surface, to 100 to 700 at a depth of 2.5 meters. The change was not gradual, but sudden and irregular. The bacteria were found to go deeper in cultivated than in uncultivated soils, but no influence of the crop upon the number of organisms could be demonstrated. A decrease from 2,564,000 bacteria at the surface to none at a depth of six feet was observed<sup>69</sup> in a stony soil, and from 524,500 at the surface to 5,800 at a depth of 1.5 meters in a wet meadow soil. Clover soils contained 6,000,000 bacteria at a depth of 20 cm. and 1,500,000 at 50 cm. depth.<sup>70</sup> A change from 8,000,000 bacteria per gram of soil at the surface to sterility at a depth of one meter was also recorded.<sup>71</sup> The numbers of bacteria usually increase from the surface to a depth of 4 to 6 inches;<sup>72</sup> this is followed by a decrease to a depth of 24 inches in humid soils. The maximum numbers were found at six inches of depth;<sup>73</sup> in some cases<sup>74</sup> the maximum was found at a depth of 4 inches, where the greatest numbers of organisms are present followed by a more or less

<sup>67</sup> Proskauer, 1882 (p. 12).

<sup>68</sup> Fränkel, 1887 (p. 19).

<sup>69</sup> Reimers, J. Ztschr. Hyg. 7: 319-346. 1889.

<sup>70</sup> Caron, A. Landw. Vers. Sta., 45: 401-418. 1895.

<sup>71</sup> Stoklasa, J. and Earnest, A. Centrbl. Bakt. II, 14: 723-736. 1905.

<sup>72</sup> Chester, F. D. Del. Agr. Exp. Sta. Bul. 65. 1904.

<sup>73</sup> King, W. E. and Doryland, C. J. T. Kans. Agr. Exp. Sta. Bul. 161. 1909.

<sup>74</sup> Brown and Smith, 1912 (p. 29).



regular drop. Soils under shade (orchard, forest, meadow) have the highest numbers of bacteria at a depth of 1 inch, then the numbers decrease with depth, while soils exposed to the sun have the highest numbers at a depth of 4 inches.<sup>75</sup>

A study<sup>76</sup> of the distribution of bacteria in different soil horizons has revealed the fact that the numbers do not decrease gradually but by a series of drops as one passes from one subhorizon to another, the greatest numbers being found, in podsol soils, in the  $A_0$ - $A_1$  horizon.

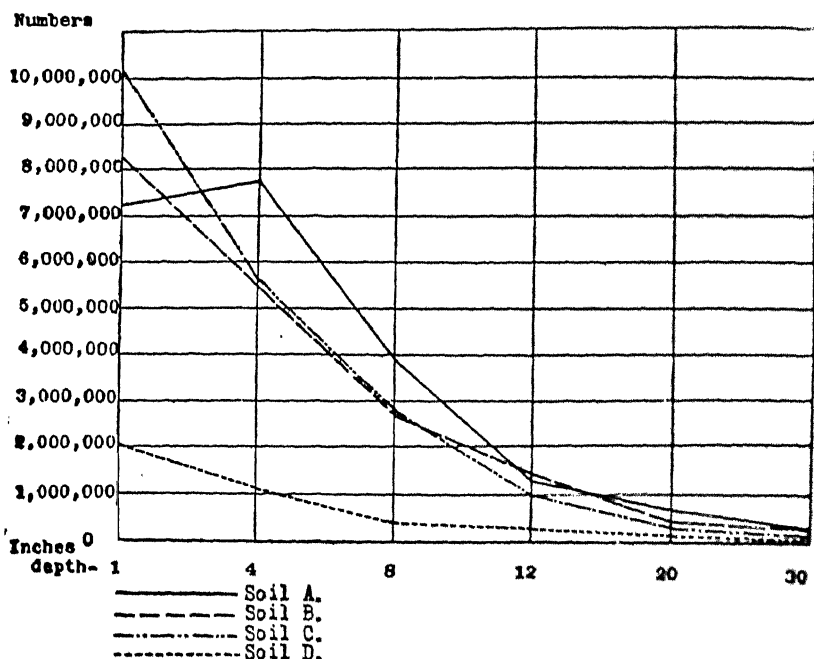


FIG. 2. Numbers of bacteria at different depths of soil, on the average of several determinations throughout the year: A, rich garden soil; B, cultivated orchard soil; C, clay soil, under timothy; D, acid forest soil (from Waksman).

In the case of arid soils, the microorganisms are found to penetrate much deeper into the subsoil layers, the activities of the organisms continuing in some cases undiminished through six feet of soil. The num-

<sup>75</sup> Waksman, S. A. *Soil Sci.*, 1: 363-380. 1916.

<sup>76</sup> Rasumov, A. S. and Remezov, N. P. *Pedology*, 24: 137-159. 1929; Brown, P. E. and Benton, T. H. *Res. Bul. 132, Iowa Agr. Exp. Sta.* 1930.

bers of bacteria may even be greater at a depth of 24 inches than at 6 inches;<sup>77</sup> a wind-blown arid soil of Arizona, of a pH 8.6 to 9.0 and with an average annual rainfall of 13 inches was found to contain 401,000 organisms at a depth of 6 inches, and 1,898,500 at a depth of 12 inches

TABLE 2  
*Distribution of soil bacteria in different depths of soil in arid regions*

DEPTH	IRRIGATED SOIL	DRY-FARM SOIL
1 foot	6,240,000	4,372,000
2 feet	1,760,000	1,267,000
3 feet	1,147,000	1,174,000

and 916,500 at a depth of 24 inches. Fifty-two per cent of the organisms developing on the plate were actinomycetes. Better aeration and presence of organic matter are no doubt largely responsible for this greater distribution of microorganisms with depth of soil in arid regions. The favorable influence of irrigation upon bacterial numbers is marked not only at the surface but also at lower depths,<sup>78</sup> as shown in table 2.

TABLE 3  
*Numbers of physiological groups of bacteria in 1 gram of soil*

	RESULTS OF HILTNER AND STÖRMER	RESULTS OF LÖHNIS, AVERAGE OF SUMMER AND WINTER VALUES	RESULTS OF MILLARD, AVER- AGE OF MAXIMUM AND MINIMUM VALUES
I. Peptone decomposing.....	3,750,000	4,375,000	75,000,000
II. Urea decomposing.....	50,000	50,000	27,500,000
III. Nitrifying.....	7,000	5,000	100,000
IV. Denitrifying.....	50,000	50,000	162,500
V. Nitrogen-fixing.....	25	388	2,500,000

*Numbers of specific physiological groups of bacteria in the soil.* In determining the numbers of specific morphological or physiological groups of bacteria in the soil, the dilution method is largely employed, as outlined above.<sup>79</sup> The number obtained indicates a certain minimum of cells of the particular organism, since often many of the cells intro-

<sup>77</sup> Lipman, C. B. Univ. Cal. Publ. Agr. Sci. 1: 7-20. 1912; 4: 113-120. 1919; Snow, L. M. Soil Sci. 21: 143-161. 1926.

<sup>78</sup> Greaves, J. E. Agricultural bacteriology. Lea and Febiger. Phila., p. 164. 1922.

<sup>79</sup> Löhnis, 1905 (p. 13); Millard, W. A. Centrbl. Bakt. II, 31: 502-507. 1911.

TABLE 4  
*The distribution of different physiological groups of bacteria in different soils (Duggeli)*

SOIL TYPE	GARDEN	VINEYARD	FIELD	MEADOW	DECIDUOUS FOREST	CONIFEROUS FOREST	MARSH LAND
Moisture content, in per cent of moist soil.....	17.9	11.1	18.1	17.0	17.6	21.2	37.2
Per cent of $\text{CaCO}_3$ .....	4.7	10.1	5.0	11.4	2.8	0	7.6
Bacteria developing on nutrient gelatin plate.....	8,400,000	3,400,000	8,100,000	8,100,000	1,900,000	1,500,000	1,500,000
Bacteria developing on nutrient agar plates.....	2,800,000	2,400,000	3,500,000	3,000,000	1,200,000	900,000	1,700,000
Bacteria growing in deep cultures of glucose agar (anaerobes).....	280,000	106,000	137,000	620,000	180,000	345,000	2,180,000
Urea decomposing bacteria.....	37,000	23,500	8,500	5,200	20,000	8,800	2,500
Denitrifying bacteria.....	830	1,720	400	850	230	380	370
Pectin decomposing bacteria.....	535,000	85,000	70,000	235,000	20,500	810,000	3,700
Anaerobic butyric acid bacteria.....	368,000	68,500	50,300	83,500	22,000	203,000	235,000
Anaerobic protein decomposing bacteria.....	35,000	5,500	22,000	36,800	700	17,000	2,000
Anaerobic cellulose decomposing bacteria.....	367	367	350	367	0.8	17.7	1.1
Aerobic nitrogen-fixing bacteria.....	2,350	537	1,885	15	17	0	17
Anaerobic nitrogen fixing bacteria.....	5,500	2,050	700	370,000	517	2,020	67
Nitrifying bacteria.....	880	3,354	1,701	37	0	0	34

duced into the specific medium may fail to develop. Hiltner and Störmer found that a soil, which gave by the gelatin plate 1,270,000 bacteria, contained a much greater number of bacteria, when determined by the dilution method.

Although by the plate method the number of *Azotobacter* cells present in the soil (when they are present at all) is rather small, amounting to only a few hundreds (800 to 1000)<sup>80</sup> or a few thousands<sup>81</sup> per gram, the direct microscopic examination reveals a great abundance of these organisms in soils properly limed and buffered. *Clostridium pastorianum* may be found in numbers ranging from 1 to 5 millions per gram of soil. In poorly buffered and in acid soils, *Azotobacter* is absent altogether and its place is taken by the *Clostridium*, as shown later.

TABLE 5

*Influence of reaction upon certain specific physiological groups of bacteria (Wilson)*

SOIL REACTION	BACTERIA IN 1 GM. OF SOIL			
	Ammonia-oxidizing bacteria	<i>Bacterium radiculicola</i>		
		<i>Medicago</i>	<i>Trifolium</i>	<i>Vicia</i>
pH				
6.2	1,000	None in 5 gm.	100,000	1,000
6.4	3,500	Positive in 5 gm.	100,000	1,000
6.6	6,280	10	100,000	1,000
6.8	25,000	1,000	100,000	10,000
7.0	35,000	1,000	1,000,000	10,000

Solid media may also be made highly selective in nature and are frequently more suitable than liquid media. The use of starch agar for determining the numbers of organisms in the soil capable of utilizing starch has been suggested,<sup>82</sup> for example.

A detailed report of the abundance of various physiological groups of bacteria in different soils is given in tables 3 and 4, the latter based upon three determinations made by Dügge<sup>83</sup> in 1920, using two samples for each determination. The numbers of various specific bacteria in

<sup>80</sup> Truffaut, G. and Bezssonoff, N. Compt. Rend. Acad. Sci. 172: 1319-1324. 1921; 173: 868-870. 1921.

<sup>81</sup> Rasumov, A. S. Trans. Institute of Fertilizers, No. 28, Moscow. 1925; See also Greig-Smith, R. Centrbl. Bakt. II, 34: 227-229. 1912.

<sup>82</sup> Hoffman, C. Centrbl. Bakt. II, 34: 386-388. 1912.

<sup>83</sup> Dügge, M. Schweiz. Ztschr. Forstwes. 1923; Landw. Vorträge. Verl. Huber. 1921. H. 3.

soil depend frequently to a large extent upon the reaction of the soil, as shown in table 5.<sup>84</sup>

*Summary.* The use of the plate method for determining the abundance and nature of soil bacteria was introduced at the very beginning of development of our knowledge of soil microorganisms. This method met later with considerable criticism, since it does not give the desired information concerning the nature of soil organisms and it overlooks all but a small portion of the total number of bacteria and other organisms in the soil. The microscopic method which was introduced for the study of the soil bacteria, showed that the soil contains much larger numbers than one could ever expect to find by the plate method. However, even Conn who first introduced the microscopic method came to the conclusion that it should supplement but not replace the plate method.

Each method gives only a relative estimate of the nature and abundance of the bacterial population of the soil; an accurate count cannot be obtained by either of the two methods. The plate excludes those organisms which do not grow upon the specific medium used; the microscope does not allow a sufficient separation between small bacteria and small particles of organic matter, while some cells are not made visible due to mechanical reasons. The plate method allows a much better classification of the organisms that develop upon it than one could hope to obtain by the use of the microscope. As a result of these considerations, both the plate and microscopic methods should be used for making a qualitative and quantitative study of bacteria in soil.

<sup>84</sup> Wilson, J. K. Proc. First Int. Congr. Soil Sci. 3: 14-22. 1928.

## CHAPTER II

### NUMBERS OF FUNGI, ALGAE AND PROTOZOA IN SOIL

*Numbers of fungi in the soil.* Fungi exist in the soil both in the form of mycelium and spores. A colony on the plate represents either a spore or a piece of mycelium in the soil. The mycelium of the fungi forms a fine network around the soil particles; it is very sensitive to drying and does not always break up into fine particles each of which would develop into a colony on the plate. As a result of this, neither the plate method nor the direct microscopic method can give any fair idea of the distribution of fungus mycelium in the soil. A high plate count of fungi may merely indicate a high sporulating capacity of certain fungi and *vice versa*. Although it has been definitely demonstrated<sup>1</sup> that the normal fungal population in the soil is present extensively in the mycelial condition, the question is frequently asked to what extent does the plate count represent the actual abundance of fungi in the soil.

Brierley<sup>2</sup> found that the plate count of fungi is open to considerable criticism: 1. the slow growing Basidiomycetes are almost all eliminated and are not found among the colonies developing on the plate; 2. the same is true of various slow growing Ascomycetes and Fungi Imperfecti; 3. some of the Phycomycetes require special technique for isolation and do not develop on the plate. Most of the published lists of fungi found in the soil represent only a fraction of the total fungous population of the soil.

There is no basis for comparing the relative abundance and potential activity between the bacterial and fungus flora of the soil; for example, 1000 colonies of fungi may indicate a greater activity than 1,000,000 colonies of bacteria, when a certain process, such as cellulose or protein decomposition is studied. However, if the 1000 fungus colonies represent inactive spores, the activity of the organism may be questioned.

With these limitations in mind and in view of the fact that most of the determinations of numbers of fungi have been made by the use of high

<sup>1</sup> Waksman, S. A. *Science*, **44**: 320-322. 1916; McLennan, E. *Ann. Appl. Biol.* **15**: 95-109. 1928.

<sup>2</sup> Brierley, W. B., Jewson, S. T., and Brierley, M. *Proc. First. Int. Congr. Soil Sci., Washington (1927)*, **3**: 48-71. 1928.

dilutions and on the bacterial agar plate, it is questionable to what extent many of the results obtained in the past actually represent even relative soil conditions. In general, the soil was found<sup>3</sup> to harbor between 30,000 and 900,000 fungi (spores and pieces of mycelium) per gram, depending on the type of soil and treatment. Others<sup>4</sup> reported a range of 42,000 to 131,000 fungi per gram of soil when plated out on three different media, with a ratio between the fungi and bacteria developing on the plate as 1:40 or 1:50. Whenever possible, these results should be checked up by microscopic observations.

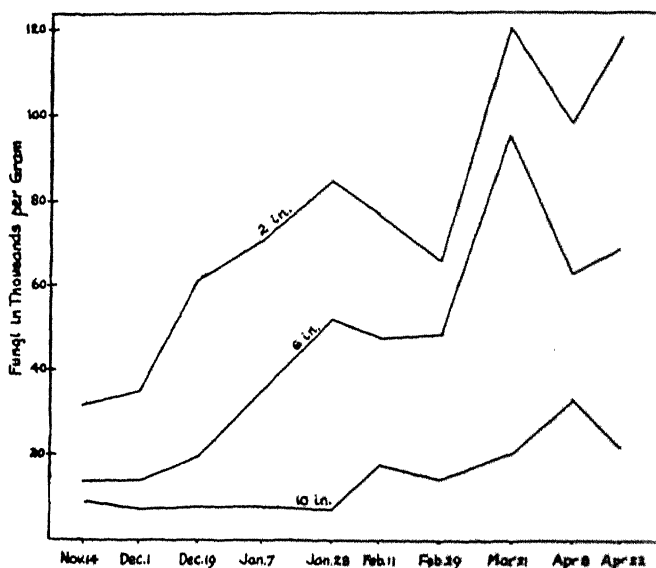


FIG. 3. Variation of numbers of fungi at different seasons of the year and different depths of soil (from Lochhead).

An acid medium similar to the one described above, combined with a low dilution, allows the determination, with a fair degree of accuracy, of the relative abundance of fungi in the soil. The more acid the soil is, the greater is the number of fungi relative to the number of the other soil microorganisms. Figure 3 illustrates the changes in the number of fungi during the year and at different depths and fig. 4 shows the in-

<sup>3</sup> Waksman, S. A. *Soil Sci.* **2**: 103-155. 1916; **3**: 585-589. 1917; *Ecology*, **5**: 54-59. 1924.

<sup>4</sup> Brown and Halversen, 1919 (p. 30).

fluence of soil treatment upon the relative numbers of bacteria and fungi in the soil, as determined by the plate method.

Among the factors which control the abundance of fungi in the soil, the reaction occupies a prominent place. Although an acid medium of pH 4.0 is used for determining the abundance of fungi in soil, since at

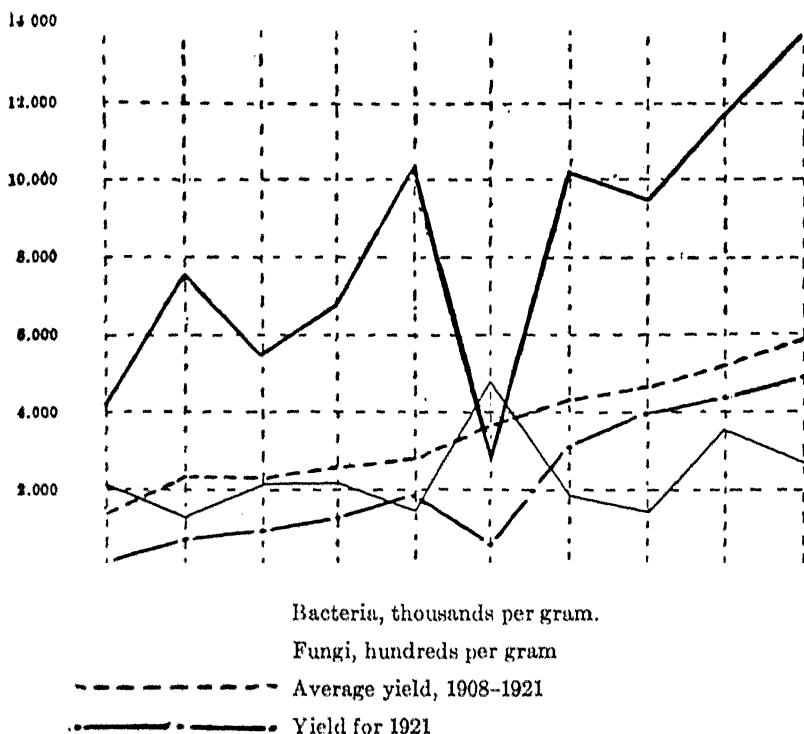


FIG. 4. Influence of soil treatment upon the numbers of bacteria and fungi in the soil: 7A and 7B, no fertilizer or manure; 4A, 19A, 19B, receiving only mineral fertilizers (phosphate and KCl); 11A and 11B, ammonium sulfate and minerals; 9A, sodium nitrate and minerals; 5A, stable manure and minerals; the B plots limed in 1908, 1913, 1918 (after Waksman and de Rossi).

that reaction the bacteria are largely eliminated, the optimum reaction for the growth of fungi lies at pH 4.5–5.5. The lower the acidity of the soil the less is the number of fungi and the greater is the number of actinomyces and bacteria. The influence of reaction of a series of plots under different fertilizer treatment upon the abundance of fungi, as determined by the plate method, is illustrated in table 6.



When the acidity of the soil becomes too high, the numbers of fungi diminish rapidly, as shown in table 7.<sup>5</sup>

TABLE 6

*Influence of liming and fertilization upon the numbers of fungi in the soil*

SOIL TREATMENT	pH	NUMBER OF FUNGI PER GRAM
Unfertilized.....	4.8	65,500
Lime alone.....	6.4	16,900
Minerals.....	5.6	45,600
Minerals + lime.....	6.4	26,300
Manure and minerals.....	5.6	91,000
Manure, minerals and lime.....	7.0	29,400
NaNO <sub>3</sub> and minerals.....	5.8	45,900
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> and minerals.....	4.6	107,900
Manure, NaNO <sub>3</sub> and minerals.....	5.8	87,600

The abundance of organic matter and variations in rainfall<sup>6</sup> are among the other factors which are found to influence markedly the development of fungi in the soil. All these counts were based upon the use of the plate method. When the microscopic method is employed, considerably larger numbers of fungi, both in the spore and mycelial stages, are found, as shown previously (p. 11).

TABLE 7

*Influence of reaction on the numbers of bacteria and fungi in one gram of an acid peat soil*

REACTION pH	BACTERIA		
	Total	Per cent of spores	
5.2	740,000	16	210,000
4.6	360,000	11	180,000
4.2	205,000	15	200,000
4.0	95,000	30	110,000
3.6	36,000	91	54,000
2.8	14,000	100	34,000

*Numbers of actinomyces in the soil.* Those media (p. 15) which are best adapted for the growth of bacteria are also well adapted for the growth of actinomyces, and their numbers can be determined on the

<sup>5</sup> Drewes, K. Centrbl. Bakt., II, 76: 114-121. 1928.

<sup>6</sup> Dixon, D. Austral. Jour. Exp. Biol. Med. Sci. 5: 223-233. 1928.

same plate used for bacterial numbers. The incubation period for counting actinomyces should be at least 7 days and, if possible, 14, days, at 25° to 30°C. If the reaction of the media favorable for the study of numbers of the three groups of soil organisms is compared, the optimum for actinomyces is usually found to be pH 7.0 to 7.5, for bacteria pH 6.5 to 7.0, for fungi pH 4.0. Of course, these are not the optima for the growth of the particular organisms in pure culture.

The actinomyces are, next to the bacteria, the most abundant group of organisms in the soil, as far as forms developing on the plate are concerned. Since a colony arises from a conidium, a chain of conidia or a piece of vegetative mycelium, larger numbers may not necessarily indi-

TABLE 8  
*Bacteria and actinomyces at various depths*  
Average of three New Jersey soils

DEPTH	BACTERIA		ACTINOMYCES	
	Numbers in 1 gram	Per cent	Numbers in 1 gram	Per cent
<i>inches</i>				
1	7,340,000	90.8	743,000	9.2
4	5,300,000	85.0	933,000	15.0
8	2,710,000	81.6	612,000	18.4
12	950,000	79.9	239,000	20.1
20	259,000	51.3	246,000	48.7
30	124,000	34.6	240,000	65.6

cate more abundant vegetative growth, but a larger abundance of conidia.

Hiltner and Störmer observed an increase in the numbers of actinomyces in the autumn, relative to the other groups of microorganisms, due to the increase of the content in undecomposed organic matter in the soil. They were found to form in the spring 20 per cent, in the fall 30 per cent, dropping in the winter to 13 per cent, of the total soil microbial flora developing on the plate. The addition of stable manure, due to its straw content, results in an increase in the numbers of actinomyces.

Loam soils contain<sup>7</sup> a higher number of actinomyces than other soils, sandy soils coming last; a proportionate increase of these organisms was

<sup>7</sup> Fousek, A. Mitt. Landw. Lehrkan. K. K. Hochschule Bodenk. Wien, 1: 217-244. 1913; Rao, M. G. and Subrahmanyam, V. Jour. Ind. Inst. Sci. 18: 253-273. 1930.

found in the fall (27 to 35 per cent) over the spring (18 to 23 per cent), but no reduction was observed in the winter. Cultivation of the soil effected a decrease in the numbers of actinomyces. These organisms are also present abundantly in forest soils (24 to 27 per cent of the flora) and on the roots of different plants, particularly on grass and legume roots, the upper cell layers of which have died down.

The soil microflora developing on the plate may consist of as many as 40 per cent of actinomyces; the total number of these organisms was reported to be as high as 12 to 14 millions per gram of soil.<sup>8</sup> Sod soils

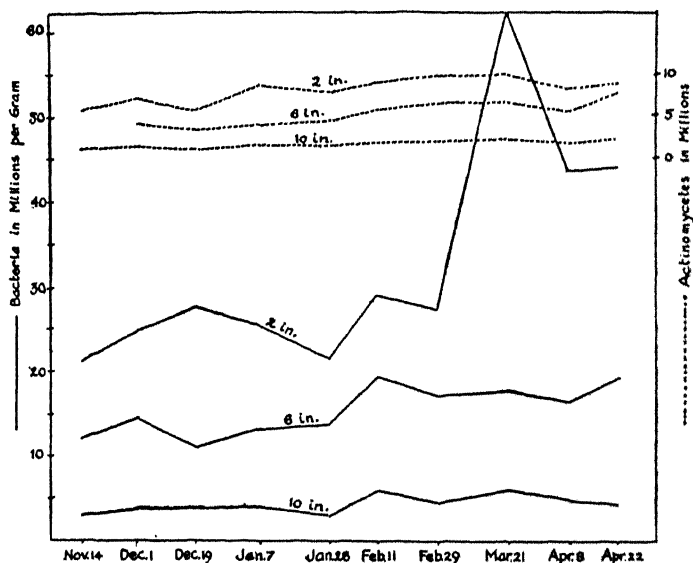


FIG. 5. Variation of numbers of bacteria and actinomyces at different seasons of the year and different depths of soil (from Lochhead).

contain larger numbers of actinomyces than cultivated soils and it was suggested, therefore, that they may play an active part in the decomposition of organic matter in the soil. The numbers of actinomyces decrease regularly with depth, but they increase in proportion to the other microorganisms, so that at a depth of 30 inches they form 65.6 per cent of the total microbial flora developing on the agar plate.<sup>9</sup>

<sup>8</sup> Conn, H. J. Jour. Amer. Soc. Agron. 5: 218-221. 1913; Jour. Bact. 1: 197-207. 1916; N. Y. State Agr. Exp. Sta. Tech. Bul. 60; Krainsky, A. Centrbl. Bakt. II, 41: 649-688. 1914.

<sup>9</sup> Waksman, S. A. and Curtis, R. E. Soil Sci. 1: 99-134. 1916.

Similar results were obtained by other investigators.<sup>10</sup> It is not known whether this is due to the greater resistance of these organisms to the lack of oxygen, to the washing down of the conidia, or to some other cause. Since these organisms are very sensitive to acidity and to an excess of moisture, acid soils and water-logged soils contain a minimum number of actinomyces. Peat bogs, even of a low acidity, contain actinomyces only at or near the surface. However, when these bogs are drained, and limed in the case of the highly acid peats, the actinomyces develop in very large numbers.

The actinomyces play an active part in the decomposition of soil organic matter, heavy soils and soils rich in undecomposed organic matter, especially when well limed or buffered, contain high numbers of actinomyces, in comparison with the bacteria of the same soil.

TABLE 9

*Influence of soil treatment on the number of actinomyces in the soil*

TREATMENT OF SOIL	ACTINOMYCES PER GRAM	PER CENT OF FLORA DEVELOPING ON PLATE	SOIL REACTION pH
Unfertilized.....	1,150,000	27.7	4.6
Lime alone.....	2,410,000	31.6	6.4
Minerals*.....	1,520,000	22.7	5.5
Manure and minerals.....	2,920,000	24.5	5.4
Minerals and ammonium sulfate.....	370,000	12.1	4.1
Minerals, ammonium sulfate and lime...	2,520,000	26.5	5.8
Minerals and sodium nitrate.....	2,530,000	25.0	5.5

\* 320 pounds of KCl and 640 pounds acid phosphate per acre every year.

The addition of manure<sup>11</sup> as well as of different other forms of undecomposed organic matter greatly stimulates the development of actinomyces and may even result in an increase in their relative abundance, in comparison with the other organisms developing on the plate. Among the other most important factors influencing the total and relative abundance of actinomyces in the soil is the soil reaction; the less acid the soil, the higher is the relative number of actinomyces. The influence of soil treatment upon the development of these organisms in the soil is shown in table 9. Jensen<sup>12</sup> reported that the highest numbers

<sup>10</sup> Lochhead, 1924 (p. 30).

<sup>11</sup> Hiltner, L. Jahrb. Ver. angew. Bot. for 1907, 5: 200-222. 1908; Bright, J. W. and Conn, H. J. N. Y. Agr. Exp. Sta. Tech. Bul. 67. 1919.

<sup>12</sup> Jensen, H. L. Soil Sci. 30: 59-77. 1930.

of actinomyces are found in soils of a pH 6.8-8.0. The per cent of actinomyces to the total number of colonies developing on the plate ranged from 0 (in the case of acid peat soils) to 73.

*Numbers of algae in the soil.* Algae are present in the soil both in a vegetative condition and in the form of resting spores. The cells of many species are surrounded by a layer of mucilage which makes an exact determination of their numbers very difficult. The dilution number may be employed for this purpose. A nutrient solution is used for the preparation of the dilutions. Five cubic centimeter portions of the final dilutions are added to test-tubes containing 15-gram portion of cleaned, sterilized sand. The inoculated tubes are placed in closed glass vessels and exposed to sunlight. The presence of specific organisms is determined at the end of one month by microscopic examination.

The algae are distributed quite uniformly within the upper few inches of soil. The numbers are usually high in the top inch; they diminish

TABLE 10  
*Abundance of algae at different depths of soil*

DEPTH	ALGAE PER GRAM OF SOIL
Surface 1 inch	16,000
2 inches	10,000
4 inches	28,000
6 inches	4,000

in the second and third inches and may increase again in the fourth inch; below that depth, the numbers drop rapidly, as shown in table 10.<sup>13</sup> In the subsoil the majority of the cells exist in the form of resting spores. Various species differ greatly in this respect. Some species grow as well on the surface of the soil as at lower depths, while others grow more abundantly near the surface, as shown in table 19.

When conditions are not favorable, as after a severe frost, the numbers diminish rapidly to even less than 100 cells per gram in the top inch of soil. Drying of soil also reduces considerably the numbers of algae.

*Numbers of protozoa in the soil.* The protozoa exist in the soil both in an active stage and in a cyst condition. The methods employed for the study of these organisms should give more or less accurate information concerning their numbers in the soil in both of these two stages. It is sometimes essential to be able to count protozoa in culture solutions.

<sup>13</sup> Bristol-Roach, B. M. Proc. First. Int. Congr. Soil Sci. 3: 30-38. 1928.

This can be done in several different ways. The most common method consists in placing a drop of a known volume of the protozoan suspension under the microscope and counting the number of protozoa. The use of a slimy colloidal solution for the purpose of reducing the motility of protozoa such as a 2 per cent gelatin solution or various gums, has been suggested.<sup>14</sup> (2) The standard loop devised for bacteria may be employed<sup>15</sup> for counting of protozoa. (3) The agar plate method has also been recommended.<sup>16</sup>

None of these methods is very suitable for the determination of the number of protozoa in the soil itself, although the first two can be used for the examination of soil for the presence of living protozoa. For an accurate determination of the total number of protozoa in the soil, including both the active forms and the cysts, the dilution method is most appropriate.<sup>17</sup> The method consists in diluting the soil with sterile tap water, then placing 1 cc. portions of the various dilutions into sterile culture media, incubating, examining the cultures at periodic intervals (5, 12 and 20 days) and determining the highest dilution at which growth takes place. The number of protozoa is thus found to lie between this and the next higher dilution at which growth did not take place. The dilutions can be made narrower and the number of protozoa determined with a greater degree of accuracy. This method can also be modified so as to give not only the total number of protozoa in the soil, but also the number of active forms and cysts.<sup>18</sup>

The soil is passed through a 3 mm. sieve and two 10-gram samples are taken. The total number of protozoa (active forms plus cysts) is determined, in one sample, while the other sample is used for the determination of cysts. Taking care to agitate the flask while the liquid is being transferred, the following dilutions are prepared: 1:10, 1:100, 1:1,000, 1:2,500, 1:5,000, 1:7,500, 1:10,000, 1:25,000, 1:50,000, 1:75,000, 1:100,000.

Nutrient agar is poured into a series of sterile Petri dishes. When the medium has solidified, the dishes are inoculated in pairs with 1 cc. of each dilution. The same pipette may be used when one begins with the highest dilution and goes back to the lowest. The plates are incubated at 18° to 20° for 28 days and examined

<sup>14</sup> Statkewitsch, P. *Arch. Protistenk.* 5: 17-39. 1905.

<sup>15</sup> Müller, P. T. *Arch. Hyg.* 75: 189-223. 1912; Koch, G. P. *Jour. Agr. Res.* 4: 511-559. 1915; 5: 477-488. 1915; *Soil Sci.* 2: 163. 1916.

<sup>16</sup> Killer, J. *Centrbl. Bakt.* II, 37: 521-534. 1913.

<sup>17</sup> Rahn, O. *Centrbl. Bakt.* II, 36: 419-421. 1913; Cunningham, A. *Jour. Agr. Sci.* 7: 49-74. 1915; Sherman, J. M. *Centrbl. Bakt.* II, 41: 625-630. 1914; also 1916 (p. 47).

<sup>18</sup> Cutler, D. W. *Jour. Agr. Sci.* 10: 135-143. 1920.

after 7, 14, 21, and 28 days. A little sterile water is added to the plates, in case they begin to dry out. The examination is made by scraping off a little of the surface of the plate, placing on a slide under the microscope and examining with low and high power.

The second 10-gram sample of soil is treated with sufficient 2 per cent HCl, over night, to neutralize the carbonate present in the soil and still leave an excess of unchanged 2 per cent HCl. This kills all the active forms leaving the cysts unharmed. The number of protozoa is then determined by the same dilution method. The number of cysts subtracted from the total number of organisms, obtained in the first count, gives the number of active protozoa per gram of soil.

Fisher<sup>19</sup> investigated the theory of estimation of microorganisms by the dilution method and found that a very high efficiency can be thus obtained (87.71 per cent). By the use of a special table, the number of protozoa can be calculated, when the number of negative plates is known. By using 26 plates and a series of dilutions ranging from  $\frac{1}{2^5}$  to  $\frac{1}{102,400}$ , the following results were obtained:<sup>20</sup>

NUMBER OF STERILE PLATES	PROTOZOA PER GRAM	NUMBER OF STERILE PLATES	PROTOZOA PER GRAM
1	110,000	14	640
2	59,000	15	450
3	36,000	16	320
4	23,000	17	230
5	16,000	18	160
6	11,000	19	110
7	7,600	20	79
8	5,300	21	56
9	3,700	22	38
10	2,600	23	25
11	1,800	24	15
12	1,300	25	6.8
13	900		

For the examination of trophic protozoa, the soil sample is moistened with some sterile water, placed on a slide and examined for a minute under the microscope.

When soil or soil suspension is placed in a medium favorable for the development of protozoa, there is found a close connection between the growth of these organisms to that of the bacteria, the former always lagging behind. There is also observed a general sequence of protozoan forms, the flagellates appearing first, followed by the ciliates and later

<sup>19</sup> Fischer, R. A. Phil Trans. Roy. Soc. London. Ser. A, **222**: 309-368. 1922.

<sup>20</sup> Cutler et al., 1922 (p. 29).

by the amoebae. A detailed review of the media used for the cultivation of protozoa is given elsewhere.

Garden soils contain a great number of protozoa; amoebae develop on agar plates, even when the inoculum is only 1 mgm. of soil.<sup>21</sup> An average of about 100 ciliates and 1000 to 10,000 flagellates per gram of soil was reported by earlier workers.<sup>22</sup> Sherman<sup>23</sup> compared the numbers of protozoa in sixteen soils representing various soil types under various treatments; he found that normal fertile soil has a protozoan

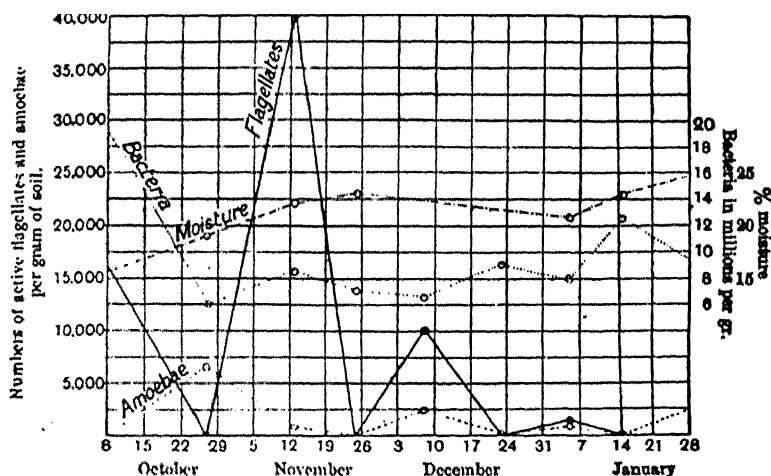


FIG. 6. Numbers of active flagellates, amoebae and bacteria in the soil, in relation to its moisture content (from Cutler and Crump).

content approximating 10,000 per gram, the flagellates constituting the greater portion; about 100 ciliates were found per gram; certain types of protozoa are active in the soil under normal and even sub-normal conditions of moisture, the active forms being probably restricted to the flagellates. Although the amoebae are also widely distributed in the soil,<sup>24</sup> their numbers have not been reported in detail due to the difficulty of their development on the common media. In soils with a moisture content not in excess of the physical optimum, protozoa exist mainly in

<sup>21</sup> Störmer, K. *Jahresb. Ver. angew. Bot.* for 1907, 5: 113-131. 1908.

<sup>22</sup> Waksman, S. A. *Soil Sci.* 1: 135-152; 2: 363-376. 1916.

<sup>23</sup> Sherman, J. M. *Jour. Bact.* 1: 35-66; 165-185. 1916.

<sup>24</sup> Martin, C. H. and Lewin, K. R. *Phil. Trans. Roy. Soc. London*, 205B: 77-94. 1914; *Jour. Agr. Sci.* 7: 106-119. 1915.



a nontrophic state.<sup>25</sup> The protozoa become active in the soil whenever there is excessive moisture present for a period of several hours. Such conditions are common, especially in rainy seasons, in poorly drained soils, in spring, etc. The protozoa then excyst and, after a period of active growth, they reproduce. When conditions become again unfavorable, they either encyst or die. Protozoa are readily observed in field ditches, furrows with standing water, etc. Since the protozoa

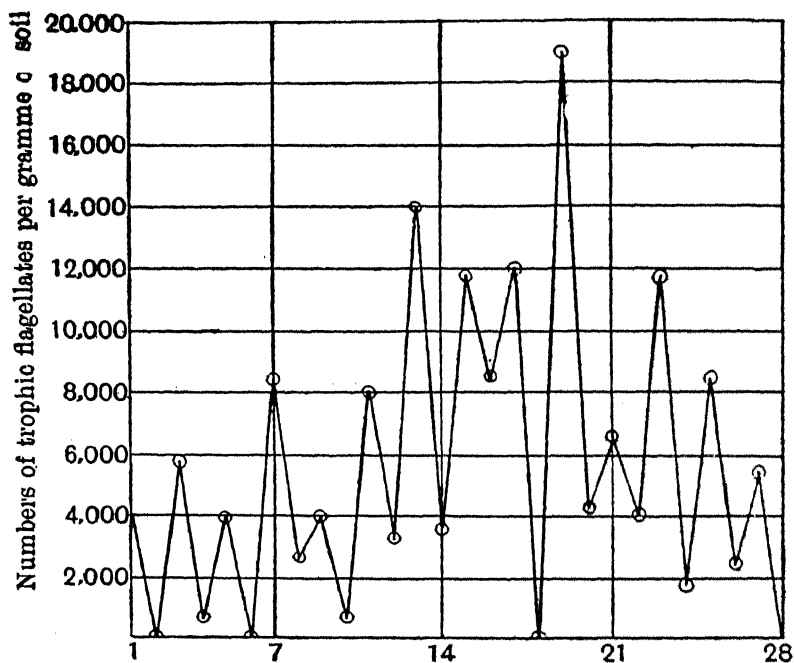


FIG. 7. Daily variation of active flagellates in the soil (from Cutler and Crump)

chiefly use bacteria as their food (perhaps also organic matter and soil extracts, especially in the case of small flagellates), they are more abundant in rich fertile soils than in poor acid soils. Their greatest numbers are concentrated in the top four or six inches of soil, where the bacteria are also at a maximum, while below twelve inches the soil is practically free from protozoa.<sup>26</sup> Isolated species may be found, usually in a cyst condition, even at very low depths.<sup>27</sup> By the use of the method above

<sup>25</sup> Fellers, C. R. and Allison, F. E. *Soil Sci.* 9: 1-25. 1920.

<sup>26</sup> Crump, L. M. *Jour. Agr. Sci.* 10: 182-198. 1920.

<sup>27</sup> Sandon, 1927 (p. 314).

described, Cutler and Crump<sup>28</sup> estimated at frequent intervals the active protozoa and cysts in Rothamsted soils, and found them to be more abundant on the manured than on the unmanured plot.

TABLE 11

*Influence of organic matter on numbers of protozoa in the soil (average of 10 counts)*<sup>28</sup>

	BROADBALK MANURED (10 PER CENT ORGANIC MATTER)			HARPENDEN FIELD (5.7 PER CENT ORGANIC MATTER)		
	C*	F	A	C	F	A
February 1-March 5, 1917.....	20	32,000	1,500	10	13,500	500
April 17-June 6, 1917.....	130	23,000	1,600	20	71,000	500
June 13-September 20, 1917.....	120	31,200	18,600	10	25,700	17,000
October 10-December 15, 1917.....	20	42,300	23,200	10	23,330	10,000

\* C = ciliates, F = flagellates, A = amoebae.

TABLE 12

*Influence of soil depth upon the numbers of protozoa*<sup>26</sup>

DEPTH	AMOEBAE PER 1 GRAM	FLAGELLATES PER 1 GRAM	CILIATES PER 1 GRAM
<i>inches</i>			
6	1,750	8,750	100
12	0	100	0
18	0	100	0

TABLE 13

*Numbers of protozoa and bacteria per gram of soil from two Broadbalk plots*<sup>28</sup>

	MANURED		UNMANURED		ACTIVE FORMS	APPROXI- MATE DIAMETER μ
	Winter total	Summer total	Winter total	Summer total		
Flagellates.....	150,000	600,000	5,500	15,000	15 to 95	7.5-15
Amoebae.....	5,000	15,000	40	2,000	per	8-22
Thecamoebae.....		1,000			cent	15
Ciliates.....	50	200			in all	20-40
Bacteria.....	10,000,000	24,000,000	4,000,000	5,000,000	cases	1-4

The numbers of protozoa and bacteria in the soil were reported to vary from day to day.<sup>29</sup> An inverse relation was found between the number of active amoebae and bacteria.

As to the influence of reaction upon the development of protozoa,

<sup>28</sup> Cutler, D. W., and Crump, L. M. Ann. Appl. Biol. 7: 11-24. 1920.

<sup>29</sup> Cutler et al., 1922 (p. 29).

Sandon demonstrated (table 14) that only testaceous rhizopods show a tendency to occur abundantly in acid soils, while alkaline soils are poor in these organisms; the other protozoa are not influenced to any considerable extent by changes in reaction.

*Summary.* The soil population consists of many millions of cells of microorganisms per single gram of soil. The microflora and microfauna of the soil comprise numerous genera and species. The abundance of the population and of its various representative groups depends upon the nature of the medium in which these organisms grow, namely the soil, upon the available food supply and upon the environmental conditions, especially air supply, temperature and reaction.

An abundance of organic matter will favor an extensive development of bacteria and fungi, which in their turn will be accompanied by

TABLE 14  
*Effect of reaction upon the numbers of species of protozoa in various soils*

RANGE OF pH	NUMBER OF CASES	AVERAGE NUMBER OF SPECIES				TOTAL SPECIES OF PROTOZOA
		Flagellates	Ciliates	Amoebae	Testaceous	
3.5-4.7	14	7.3	3.6	2.6	3.6	17.1
4.7-6.0	27	7.6	2.9	2.3	3.0	14.8
6.0-7.3	59	6.7	3.5	2.9	2.1	15.2
7.3-8.5	27	6.6	4.7	2.5	1.0	14.8
8.5-9.8	14	6.6	4.6	1.7	0.6	13.5

an abundant development of protozoa and worms. An acid reaction will favor the development of the fungi and will tend to suppress the growth of bacteria, especially of certain groups which take an active part in several important soil processes; an alkaline reaction will tend to depress the development of the fungi and will be favorable to actinomycetes and various bacteria. Insufficient aeration, as in peat bogs, will eliminate below the surface the fungi, actinomycetes and many aerobic bacteria, and will favor the development of obligate and facultative anaerobic bacteria. A high temperature will prove favorable to the development of certain specific groups of thermophilic bacteria.

In addition to the many members of the soil population which are known and have been described, the soil harbors numerous organisms which still await description. The methods of isolation of the various representatives of this population, the methods of cultivation and determination of their various functions and rôles in soil processes will be elucidated in the following chapters.

*PART B*  
ISOLATION, IDENTIFICATION, AND CULTIVATION  
OF SOIL MICROORGANISMS

*"Tota in minimis existit natura"*—MALPIGHI



## CHAPTER III

### PURE CULTURE STUDY AND CLASSIFICATION OF SOIL BACTERIA

It is almost impossible at present to make a complete study of the various types of bacteria occurring in the soil, due both to the great variety of forms and to the lack of sufficient knowledge concerning many of them. Bacteria offer but few stable characteristics which can be utilized for their separation. Classification of bacteria in general and of soil bacteria in particular is not fully satisfactory. The different systems of classification can, therefore, be merely tentative; they are based either on the physiological activities of the organisms, especially their carbon, nitrogen, and oxygen metabolism, or on their morphological relationships. In the general subdivision of the soil bacteria, the physiological system will be utilized for the greater convenience which it offers in the study of the rôle of these organisms in the soil; in the differentiation of the individual bacteria within the large groups morphological characters are utilized.

*Pure culture study.* In the case of large organisms, a pure culture corresponds to an individual. In the case of the unicellular bacteria, the transformation carried on by one cell is too small, and large numbers of organisms must take part in a reaction, before measurable changes are obtained; these individual cells, however, must be as much alike as possible. The various bacteria do not exist in the soil in pure culture, but in mixed associations; entirely different results are usually obtained under natural conditions, with the various microbiological processes completing one another, than in pure culture. This is especially true in the case of organisms which depend for their nutrients upon the activities of other organisms, as in the case of the nitrite bacteria which depend upon the various proteolytic organisms for their substrate (ammonia), and the nitrate-forming bacteria which depend upon the nitrite formers for their supply of energy source (nitrite). Antagonistic effects are often marked under natural surroundings, while they are eliminated in pure culture. Certain soil processes, such as cellulose decomposition or nitrogen-fixation, are carried on much more actively by crude than by pure cultures of the organisms concerned, due to the fact that the accompanying organisms either form intermediate or end products specially

suitable for food, or use up the deleterious waste products produced as a result of bacterial metabolism, or contribute in some other way to the process in question.

To be able to identify the various species of bacteria and determine their specific functions upon nutritive media and in the soil, it is necessary to obtain them in pure culture. A mere microscopic examination of bacteria is usually insufficient for their identification. They should be studied on artificial culture media and records made of the cultural characteristics and biochemical changes produced. Among these characteristics, we may include size, appearance and color of colony; growth upon solid and liquid media; modification of color, consistency, reaction and chemical composition of the medium; action upon sugars and proteins and enzyme formation, and influence of oxygen tension, temperature and chemical agents. Among the morphological characteristics, we may include form, size, grouping and appearance of cells, motility, spore formation, manner of reproduction, etc.

Pure cultures of bacteria and other microorganisms are often weakened, when grown on artificial culture media, or may lose altogether their capacity of producing the change which they carry on in nature, as in the case of *Azotobacter* and other nitrogen fixing bacteria. This leads to differences in results in the hands of different investigators. We are dealing here with organisms whose functions are changeable and which are very variable in their activities.<sup>1</sup> As a result of these considerations, one would hardly be justified in drawing conclusions concerning the rôle of certain bacteria in soil processes on the basis of pure culture study alone. However important it is to isolate an organism which is believed to be the causative agent of a certain process in soil, it is just as important to know what this organism does in the soil, in the presence of numerous other organisms. It becomes evident, therefore, that a knowledge of soil bacteria and their rôle in soil processes should not be based on the study of pure cultures alone. To illustrate this phenomenon, one may only cite the case of *Bact. radicicola*, the organism responsible for the formation of nodules on the roots of leguminous plants. While it is living in the nodule, in association with the tissues of the host, this organism is responsible for the fixation of large quantities of atmospheric nitrogen. Recent evidence points to the fact that in pure culture this organism is practically unable to bring about any fixation of nitrogen, even in the presence of favorable sources of energy.

<sup>1</sup> Pringsheim, H. Die Variabilität niederer Organismen. Berlin. J. Springer. 1910.

In obtaining pure cultures of a number of soil bacteria, especially those producing certain biochemical changes when grown in the presence of specific substances, the selective or accumulative culture method used extensively by Beijerinck,<sup>2</sup> Winogradsky and others may be employed. The first step made in the isolation of a particular organism consists in adding some of the material (soil or manure) to a liquid medium containing nutrients especially adapted to the growth of that particular organism. This allows the accumulation of the organism in question and eliminates largely the other accompanying forms. By transferring repeatedly to the same sterile medium, an enrichment culture is obtained. Theoretically one species should develop under ideal enrichment conditions; actually, a number of strains may be found in the culture, which, on separation, are frequently mistaken for different species.

The direct method of isolation suggested by Winogradsky<sup>3</sup> may also be employed. This consists in adding a specific nutrient to a silica gel plate, containing the necessary nutrients, and inoculating with particles of soil. Only the organisms capable of acting upon the specific substrate will develop on the plate, which can then be readily isolated.

In the case of the majority of soil bacteria (especially the heterotrophic forms), both aerobic or anaerobic, the plate method will prove convenient for the isolation of the individual organisms from colonies. The selective culture method in most cases and the plate method in some cases yield only crude cultures of the organisms. For biochemical studies and especially for the study of life cycles of bacteria, it is advisable to obtain single-cell cultures of the organisms.<sup>4</sup> In most instances this is accomplished by the dilution method, which may be accompanied by the transfer of a single cell by the India ink<sup>5</sup> method or by means of a capillary pipette.<sup>6</sup> The first consists in mixing a highly diluted cul-

<sup>2</sup> Richter, O. *Die Bedeutung der Reinkultur*. Borntraeger. Berlin. 1907; Stockhausen. F. *Oekologie, Anhäufungen nach Beijerinck*. Berlin. 1907.

<sup>3</sup> Winogradsky S. *Compt. Rend. Acad. Sci.* **180**: 711-716. 1925.

<sup>4</sup> Löhnis, F. *Studies upon the life cycles of the bacteria*. I. *Mem. Nat. Acad. Sci.* **16**: 1921.

<sup>5</sup> Burri, R. *Centrbl. Bakt.* **II**, **20**: 95-96. 1908; *Das Tusche-punktverfahren*. G. Fischer. Jena. 1909.

<sup>6</sup> Barber, M. A. *Kansas Univ. Science Bull.* **4**: no. 1 48 p. 1907; *Jour. Inf. Dis.* **5**: 379. 1908; **8**: 348. 1911; *Philippine Jour. Sci. B.* **9**: 307-360. 1914; *Jour. Exp. Med.* **32**: 295. 1920; Shouten, S. L. *Ztschr. Wissensch. Mikrosk.* **22**: 10, 1907; **24**: 258. 1907; Hecker, F. *Jour. Inf. Dis.* **19**: 305. 1916; *Hort. Jour. Hyg.* **18**: 361. 1920; Topley, W. W. C., Barnard, J. E. and Wilson, G. S. *Jour. Hyg.* **20**: 221-226. 1921; Malone, R. H. *Jour. Path. Bact.* **22**: 222. 1918.



ture with sterile India or China ink, placing pin-point droplets of the mixture upon the surface of sterile agar or gelatin, covering with sterile cover slips, examining with high-power objective for the presence of a single cell, incubating, and finally transferring or lifting the cover slip with adhering bacterial cell and inoculating into sterile medium. The second method consists in drawing up individual cells by means of the Barber capillary pipette and transferring them to specific sterile liquid media. The micro-manipulator of Chambers<sup>7</sup> may also be utilized for this purpose.

In view of the fact that the bacteria live and act in the soil in mixed culture, important information is obtained not only from the study of pure cultures, but also from mixed cultures, either crude or artificially prepared.

The Chart of the Society of American Bacteriologists which includes a study of the important morphological, physiological and cultural characteristics of the organisms, can be used only in the description of the heterotrophic bacteria requiring combined nitrogen, especially the spore-forming organisms.<sup>8,9</sup>

*Differentiating characters of bacteria.* The size of the organisms is a variable factor with rather large limits of variation which depend upon the nutrient media in which the cells are grown and various environmental conditions.<sup>10</sup> However, in the case of the spore-forming bacteria, the size of the spore is of great diagnostic value. Motility of bacteria has been utilized in the classification of Migula but the constancy and value of this character have been questioned by Lehmann and Neumann who found the presence or absence of spore production by bacteria to be a more definite characteristic. The form of growth of the various organisms is also of important diagnostic value.

Although the morphological characters of the bacteria should be utilized in a scientific system of classification, the soil bacteria lend themselves readily to a general classification based upon their physiological

<sup>7</sup> Chambers, R. Jour. Inf. Dis. 31: 334-343, 344-348. 1922.

<sup>8</sup> Harding, H. A. N. Y. Agr. Exp. Sta. Tech. Bul. 13. 1910; Rahn, O. and Harding, H. A. Centrbl. Bakt. II, 42: 385-393. 1914.

<sup>9</sup> The methods used in the description of these organisms are discussed in the various Reports of the Committee on Bacteriological Technic of the Society of American Bacteriologists. Jour. Bact. 3: 115-138. 1918; 4: 107-132. 1919; 5: 127-143. 1920; 7: 107-132. 1919; Conn, H. J. et al. Ibid. 10: 315-319. 1925; N. Y. Agr. Exp. Sta. Tech. Bul. 57: 18-42. 1917.

<sup>10</sup> Löhnis, F. and Smith, N. R. Jour. Agr. Res. 6: 676-702. 1916; 23: 401-432. 1923; also Löhnis, 1921 (p. 55); Scales, F. M. Jour. Inf. Dis. 29: 591-610. 1921.

activities. Such a system is suggested here for the purpose of grouping the bacteria according to the important soil processes in which they take an active part, utilizing the more systematic classification for the discussion of the specific organisms.

*Life cycles of bacteria.* According to Löhnis, bacteria live alternately in an organized and in an amorphous or "symplastic" stage. In the latter stage the living matter which has been previously enclosed in the separate cell, undergoes a thorough mixing either by a complete disintegration of the cell wall and cell content or by a "melting together" of the contents of many cells which leave their empty cell walls behind them; it seems to be formed both of the vegetative and reproductive cells; this process is similar to autolysis, without the destruction of the living substance. The symplasm may undergo amoeboid changes or become encapsulated, giving spherical macrocysts. In the process of formation of new individual cells from the symplasm, "regenerative units" are first visible, which increase in size becoming either directly vegetative cells or "regenerative bodies." The latter become, by germination and stretching, normal cells or return temporarily into the symplastic stage.

In addition to symplasm formation, two or more individual cells may unite directly (conjunction). All bacteria multiply not only by fission but also by the formation of gonidia, which first become regenerative bodies or exospores. The gonidia may either grow directly into full cells or enter the symplastic stage. Thread-like branching forms may also be produced from the symplasm. The life cycle of each species of bacteria is composed of several sub-cycle showing wide morphological and physiological differences. They are connected with each other by the symplastic stage (No. 46, Pl. VI). However, some investigators<sup>11</sup> deny the formation of symplasm by *Azotobacter*; this is looked upon as a stage of gradual autolysis of the cell membrane rather than a stage of reproduction. Henrici<sup>12</sup> demonstrated that bacterial cells undergo metamorphosis during the growth of a culture, similar to that exhibited by cells of a multicellular organism; each species presents three types of cells: a young form, an adult form and a senescent form; the variations depend upon the rate of metabolism. Others<sup>13</sup> consider

<sup>11</sup> Beauverie, J. *Compt. Rend. Acad. Sci.* 180: 1792-1794. 1925.

<sup>12</sup> Henrici, A. T. *Morphologic variation and the rate of growth of bacteria.* C. C. Thomas, Springfield, 1928.

<sup>13</sup> David, H. *Centrbl. Bakt.* II, 70: 1-29, 1927. See also Enderlein, J. *Bacterien-Cyclogenie.* W. de Gruyter. Berlin & Leipzig. 1925; Klineckowström, A. V. *Ark. Bot.* 23A: No. 12. 1931.

the bacteria to grow in certain cycles, in which several stages may show definitely varying properties not only in their morphology, but also in the biological, serological, physiological and pathological behavior. Pleomorphism in bacteriology points to the numerous deviations from the typical appearance of the bacterium within the normal life cycle and may alternate. These differences cannot be considered as mutations, as some investigators attempted to prove.

*Classification of bacteria.* This is not the place to discuss the value of the different systems of classification of bacteria. It is sufficient to call attention to the systems of Migula, Lehmann and Neumann, and to that proposed by the Committee on Classification of the Society of American Bacteriologists and extended further by Bergey. One may conveniently use here Migula's classification, with slight modifications, especially in the case of the Bacteriaceae, which are more commonly divided on the basis of spore formation.

I. Simple and undifferentiated forms, not forming any threads and not branching under normal conditions. Order EUBACTERIA.

1. Cells mostly spherical, rarely rod-shaped, COCCACEAE Zopf emend. Migula:

- (a) Division in one direction, frequent formation of chains, 1. *Streptococcus* Billroth.
- (b) Irregular division in all directions; cells occur singly, in pairs or clumps, but not in chains, 2. *Micrococcus* Cohn.
- (c) Division in three directions leading to packet formation, 3. *Sarcina* Goodsir.

Planostreptococcus, Planococcus, Planosarcina include similar but flagellated group.

2. Cells mostly rod-shaped, rarely spherical or curved, BACTERIACEAE Zopf emend. Migula:

- (a) No endospores formed, 4. *Bacterium* Cohn.
- (b) Endospores formed, 5. *Bacillus* Cohn.
- (c) Cells with polar flagella; endospores rarely formed, 6. *Pseudomonas*.

3. Cells mostly curved or spiral, rarely spherical or rod-shaped, SPIRILLACEAE Migula:

- (a) Cells comma-shaped, 7. *Vibrio* Müller emend. Löffler.
- (b) Cells rigid, spiral shaped, 8. *Spirillum* Ehrenberg emend. Löffler.
- (c) Cells flexible, spiral shaped, 9. *Spirochaeta* Ehrenberg.

II. Colorless bacteria accumulating sulfur within their cells. Order THIOBACTERIA.

III. Alga-like bacteria. Order PHYCOBACTERIA. This order comprises the family Chlamydbacteriaceae which form sheaths; these include certain of the so-called iron bacteria.

- IV. Fungus-like bacteria, rod-shaped, rarely filamentous. Order MYCOBACTERIA. The Actinomyces are frequently included in this genus.
- V. Bacterial cells enclosed in a slimy mass, forming a pseudoplasmodium-like aggregation before passing into a cyst-producing resting stage. Order MYXOBACTERIA. These include the genera *Myxococcus*, *Polyangium* and *Chondromyces*.

*Classification of soil bacteria based upon their physiological activities*<sup>14</sup>

- I. Autotrophic and facultative autotrophic bacteria, deriving their carbon primarily from the CO<sub>2</sub> of the atmosphere and their energy from the oxidation of inorganic substances or simple compounds of carbon.
1. Bacteria using nitrogen compounds as sources of energy.
  2. Bacteria using sulfur and sulfur compounds as sources of energy.
  3. Bacteria using iron (and manganese) compounds as sources of energy.
  4. Bacteria using simple carbon compounds as sources of energy.
  5. Bacteria using hydrogen as a source of energy.
- II. Heterotrophic bacteria deriving their carbon and energy from various organic compounds:
1. Nitrogen-fixing bacteria, deriving their nitrogen from the atmosphere, in the form of gaseous atmospheric nitrogen:
    - a. Non-symbiotic nitrogen-fixing bacteria:
      - (a) Anaerobic types: Butyric acid bacteria (*Bac. amylobacter*), including species of *Clostridium*, *Granulobacter*, etc.
      - (b) Aerobic types: *Azotobacter*, *Radiobacter*, *Bact. aerogenes*, *Bact. pneumoniae*, etc.
    - b. Symbiotic nitrogen-fixing (nodule) bacteria.
  2. Aerobic bacteria requiring combined nitrogen:
    - a. Spore producing bacteria.
    - b. Non-spore producing bacteria.
  3. Anaerobic bacteria, requiring combined nitrogen.

<sup>14</sup> A detailed system of classification of bacteria based on biochemical relationships has been proposed by Orla-Jensen, S. *Centrbl. Bakt.* II, 22: 305-346. 1909; see also Rahn, O. *Ibid.* 78: 1-21; 79: 321-337. 1929. Janke, A. *Österr. Bot. Ztschr.* 78: 97-128. 1929.

## CHAPTER IV

### AUTOTROPHIC BACTERIA

*The nature of autotrophic bacteria.* The autotrophic bacteria are organisms capable of obtaining their carbon from the  $\text{CO}_2$  of the atmosphere and their energy by the oxidation of inorganic substances, including simple inorganic compounds (or the elementary form) of nitrogen, sulfur, iron, hydrogen and carbon. Some of these transformations, particularly those of the nitrogen and the sulfur compounds are of great importance in the soil.

The autotrophic bacteria, or *anorgoxydants*, are characterized<sup>1</sup> by a series of physiological properties, which differentiate them sharply from the rest of the bacteria. These properties can be summarized as follows:

1. Their development in nature takes place in strongly elective mineral media, which contain specific oxidizable inorganic substances.
2. Their existence is connected with the presence of these substances, which undergo oxidation as a result of the life activities of the organisms.
3. This oxidation process supplies their only source of energy.
4. The organisms do not need any organic nutrients for structure or for energy.
5. They are almost incapable of decomposing organic matter and may even be checked in their development by its presence.
6. They use, as an exclusive source of carbon, carbon dioxide, which is assimilated chemosynthetically.

These conceptions were later modified in two respects:

1. The presence of organic matter may not prove injurious to the activities of the autotrophic bacteria. As a matter of fact, the presence of small quantities of certain organic substances may even be stimulating to some. Further, the existence of these organisms in the soil, where they carry on their life processes, takes place in the presence of soluble organic substances.

2. Only few of the autotrophic bacteria are obligate, some are facultative autotrophic. The latter, as in the case of some sulfur, hydrogen

<sup>1</sup> Winogradsky, S. Centrbl. Bakt. II, 57: 1-21. 1922.

and methane bacteria, can exist both autotrophically and heterotrophically.

*Classification of autotrophic bacteria*

- A. Bacteria deriving their energy from the oxidation of simple inorganic compounds of nitrogen:
  - I. Bacteria oxidizing ammonia to nitrite (*Nitrosomonas*, *Nitrosococcus*).
  - II. Bacteria oxidizing nitrite to nitrate (*Nitrobacter*).
- B. Bacteria deriving their energy from the oxidation of sulfur or its simple compounds:<sup>2</sup>
  - I. *Endothiobacteria*, acting primarily upon  $H_2S$  and accumulating sulfur within their cells; obligate autotrophic, varying greatly in size and shape; so-called "true sulfur bacteria," occurring largely in fresh and sea waters.
    - 1. *Leucothiobacteria*, colorless organisms:
      - (a) *Beggiatoaceae*, filamentous forms, comprising three genera.
      - (b) *Achromatiaceae*, unicellular forms, comprising four genera.
    - 2. *Rhodothiobacteria*, or red sulfur bacteria, comprising 6 families with 14 genera.
  - II. *Ectothiobacteria*, minute, colorless bacteria, capable of oxidizing thio-sulfate, elementary sulfur or sulfides, but not accumulating sulfur within their cells (genus *Thiobacillus*); obligate and facultative autotrophic, occurring in dust, and especially in soil and in fresh and salt water.
    - 1. Obligate autotrophic:
      - (a) Bacteria using free atmospheric oxygen for the oxidation of sulfur:
        - (a') Bacteria growing best at a neutral or alkaline reaction, oxidizing primarily thiosulfate and precipitating sulfur outside of their cells (*Th. thioparus*).
        - (b') Bacteria growing best in highly acid media and oxidizing primarily elementary sulfur (*Th. thio-oxidans*).
      - (b) Bacteria capable of growing under anaerobic conditions, with nitrate as a source of oxygen for the oxidation of sulfur (*Th. denitrificans*).
    - 2. Facultative autotrophic and facultative anaerobic bacteria (species of Lieske and Trautwein).
- C. Bacteria deriving their energy from the oxidation of ferrous or manganous compounds:
  - I. Filamentous bacteria (*Leptothrix* and *Crenothrix*).
  - II. Minute bacteria:
    - 1. Bacteria producing long excretion filaments (*Gallionella*).
    - 2. Bacteria forming masses of coccoid or oval shaped cells (*Siderocapsa* and *Sideromonas*).

<sup>2</sup> Baas-Becking, L. G. M. Ann. Bot. 39: 613-650. 1925; Bavendamm, W. Abderhaldens Handb. Biol. Arb. Meth. 1930.

- D. Bacteria deriving their energy from the oxidation of hydrogen:
  - I. Obligate autotrophic (*Hydrogenomonas vitrea* and *H. flava*).
  - II. Facultative autotrophic:
    - 1. Aerobic (*Bac. pycnoticus*).
    - 2. Anaerobic, using nitrate as source of oxygen (*H. agilis*, *Bac. hydrogenes*):
- E. Bacteria deriving their energy from the oxidation of simple compounds of carbon:
  - I. Bacteria oxidizing methane.
  - II. Bacteria oxidizing carbon monoxide.
- F. Bacteria capable of obtaining their energy from the oxidation of selenium. Evidence limited.
- G. Bacteria capable of obtaining their energy from the oxidation of arsenites. Evidence limited.<sup>3</sup>

*Bacteria deriving their energy from the oxidation of nitrogen compounds.* Various purely chemical theories were suggested at different times to explain the process of nitrification in nature. Pasteur<sup>4</sup> was the first to indicate that the oxidation of ammonia to nitrate is accomplished by the agency of microorganisms. This view was definitely confirmed (1877) by Schlösing and Müntz,<sup>5</sup> who demonstrated that the heating of soil, otherwise capable of rapidly transforming ammonia to nitrites, to 100°C. or treating it with antiseptics (chloroform) was sufficient to prevent nitrification; when a fresh portion of soil was added to the treated soil, the power of transforming ammonia to nitrates was restored. Aeration was found to be essential to nitrification. It was obtained either by bubbling air through the medium, or by spreading the medium in a thin layer over the bottom of the container. According to Schlösing,<sup>6</sup> the quantity of oxygen consumed during the process of nitrification bears a constant ratio to the amount of nitrogen nitrified. A temperature of about 37°C. and the presence of calcium carbonate or alkaline carbonates in low concentrations (0.2 to 0.5 per cent) were found to be favorable. These investigations proved that the conditions commonly utilized in the saltpeter heaps were quite essential for the activities of the organisms; namely, (1) the presence of nitrogenous organic compounds

<sup>3</sup> Green, H. H. Fifth and Sixth Rpts. Vet. Res. Dept. Agr. Union So. Africa, 595-610. 1918; The energy reactions of autotrophic bacteria are discussed in detail by Baas-Becking, L. G. M., and Parks, G. S. *Physiol. Rev.* 7: 85-106. 1927; Starkey, R. L. In *Newer Knowledge of Bacteriology and Immunology*, p. 321-331. Chicago Univ. Press. 1928.

<sup>4</sup> Pasteur, L. *Compt. Rend. Acad. Sci.* 54: 265-270. 1862.

<sup>5</sup> Schlösing, Th. and Müntz, A. *Compt. Rend. Acad. Sci.* 84: 401. 1877; 85: 1018-1020. 1877; 86: 892. 1878; 89: 891-4, 1047-7. 1879.

<sup>6</sup> Schlösing, Th. *Compt. Rend. Acad. Sci.* 109: 423-428. 1889.

thoroughly mixed with the soil, (2) a thorough aeration of all the layers of the heap, (3) a proper moisture content kept up by moistening the heap at regular intervals, (4) the presence of bases, like calcium carbonate (or soap water). The activities were found to be noticeable at 5°C., became prominent at 12° and reached a maximum at 37°. Higher temperatures (45°) exerted an injurious effect and at 55°C. the process came to a standstill.

Warington<sup>7</sup> confirmed the biological nature of nitrification not only in soil, but also in ammoniacal solutions inoculated with soil. He studied (1) the influence of organic substances upon nitrification, (2) the abundant production of nitrites in the process of nitrification and (3) the nitrification of organic nitrogen. Ammonia could be acted upon only to a very limited extent in a liquid medium containing ammonium chloride, chalk, and sodium-potassium tartrate; when sugar replaced the tartrate, there was a decided injurious effect upon the process of nitrification, which could not be explained, since the nature of the organism was unknown. The relative amounts of nitrite and nitrate formed in the process of nitrification presented another unexplainable difficulty. As to the nitrification of organic nitrogenous substances (urine, milk, asparagine), Warington demonstrated that this has to be preceded first by their transformation into ammonia; in other words, only those substances can be nitrified which can first be converted into ammonia. It was soon found that nitrites arose from the oxidation of ammonia and not from the reduction of nitrates; Munro<sup>8</sup> distinguished ammonia formation from ammonia oxidation (nitrification) and, without suspecting the existence of the different organisms, he had a rather clear idea of the two processes.

All attempts to isolate the specific organisms concerned in the process of nitrification failed, in spite of the fact that nitrification is an important biological process not only in the soil but also in sewage purification. This was chiefly due to the fact that the proper methods were lacking. Although claims have been put forth by various investigators<sup>9</sup> that a number of organisms, some even pathogenic in nature, are able to produce nitrates, they did not receive any recognition due to improper interpretation of results. The traces of nitrates probably came from

<sup>7</sup> Warington, R. *Jour. Chem. Soc. (London)*, **33**: 44-51. 1878; **35**: 429-456. 1879; **45**: 637-672. 1884; *Chem. News*. **61**: 135. 1890; *Trans. Chem. Soc. London*, **59**: 484-529. 1891.

<sup>8</sup> Munro, J. H. M. *Jour. Chem. Soc.* **49**: 632-681. 1886.

<sup>9</sup> Heraeus, W. *Ztschr. Hyg.* **1**: 193-235. 1886.



the atmosphere and the nitrites from the reduction of the nitrates present in the medium.<sup>10</sup> The French<sup>5</sup> and English<sup>7,11</sup> investigators were primarily chemists, while the German bacteriologists<sup>9</sup> were so much under the influence of the gelatin plate method of R. Koch that the absence of growth on that medium was thought to indicate the entire absence of an organism.

Winogradsky<sup>12</sup> started out with the idea that we were dealing here with an organism of unknown properties which does not develop on the gelatin plate. Fresh from his work on the sulfur and iron bacteria (1885-1888), whereby he recognized organisms which can derive their energy from inorganic compounds, he reasoned that a source of energy so abundant as ammonia would be likely to be utilized by microorganisms. If so, the organisms concerned might show properties similar to those of organisms oxidizing other inorganic substances. The principle of elective culture was adopted, whereby conditions are made unfavorable for the development of any other bacteria, except those that are able to oxidize ammonium compounds. A medium of the following compositions was employed:

Ammonium sulfate.....	1 gram
Potassium phosphate.....	1 gram
Tap water.....	1000 cc.

Portions of this medium (100 cc.) were placed in flasks each of which contained 0.5 to 1 gram basic magnesium carbonate. The flasks were inoculated with a little soil and, when active nitrification took place, transfers were made to fresh quantities of medium. The ammonia disappeared in two weeks, while the di-phenylamine reaction for nitrates appeared in four days and reached its maximum in another three to four days. Gelatin plates made from the flasks gave various species of bacteria and yeasts, but none of them was able to nitrify. This confirmed the idea of Winogradsky that the organism in question cannot develop on gelatin. After several transfers, the observation was made that a bacterium was present on the magnesium carbonate sediment, covering it in the form of a zooglea. Under the microscope, the bacterium from the sediment was found to be regularly oval or ellipsoidal

<sup>10</sup> Winogradsky, S. *Lafar's Handb. Tech. Myk.*, 3: 132-181. 1904.

<sup>11</sup> Frankland, G. C., and P. F. *Trans. Roy. Soc. London, B*, 181: 107-128. 1890.

<sup>12</sup> Winogradsky, S. *Ann. Inst. Past.* 4: 213-231, 257-274. 1890; 5: 92-100. 1891; *Arch. Sci. Biol. St. Petersburg*, 1: 87, 127. 1892.

in nature; it was also present in the medium. In order to obtain pure cultures of these organisms two facts were considered, namely that the bacterium did not grow on the gelatin plate or in nutrient bouillon but was found abundantly on the magnesium carbonate sediment. Some of that sediment was streaked out over a gelatin plate and those particles, which remained sterile on the plate, after a few days incubation, were transferred into fresh flasks containing the ammonium medium. The final steps in the study and isolation of the bacteria concerned in the process of nitrification were the separation of the nitrite and nitrate organisms and their cultivation upon specific media.

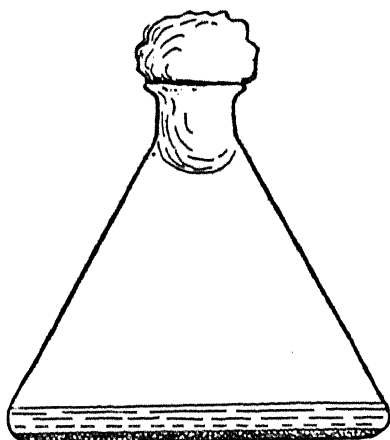


FIG. 8. Winogradsky flask for experiments on nitrification. Layer of  $\text{MgCO}_3$  on bottom of flask and mineral salt solution above it (after Omeliansky).

For the growth of the nitrite and nitrate forming bacteria, a thorough aeration of the culture is essential. Flasks of large diameter (12 cm.) with flat bottoms, in which the liquid formed only a shallow layer (less than 1 cm. in height) were used. Boulanger and Massol<sup>13</sup> employed scoria and allowed the liquid to reach only half the height of the scoria layer, which was moistened at regular intervals, by the shaking of the flask; this hastened the process of nitrification. Still more intensive nitrification was obtained by allowing the ammoniacal solution to flow through peat and carbon black inoculated with nitrifying organisms.<sup>14</sup>

<sup>13</sup> Boulanger, E. and Massol, L. *Ann. Inst. Past.* 17: 492-515. 1903; 18: 181-196. 1904. *Compt. Rend. Acad. Sci.* 140: 687. 1905.

<sup>14</sup> Müntz, A. and Lainé, E. *Compt. Rend. Acad. Sci.* 142: 1239-1244. 1906; also *Ann. Sci. Agron. Ser. 3*, 2: 287-395. 1906.

Ignited soil placed in flat bottom Fernbach flasks, combined with a slow rotary movement of the culture, gave intensive nitrification.<sup>15</sup>

An alkaline reaction is essential for nitrite formation. In the absence of basic carbonates, only ammonium carbonate is oxidized; in the presence of sodium, magnesium or calcium carbonate, free ammonia as well as the sulfate, phosphate and chloride of ammonium can nitrify.<sup>16</sup> This is due to the fact that the optimum reaction for the organisms is on the alkaline side of neutrality (pH 7.0 to 8.0). The presence of the base in the medium is essential as a neutralizing agent to prevent the reaction of the medium from becoming too acid, due to the formation of nitric and sulfuric acids from the oxidation of the ammonium salt.

In addition to the medium given above, two other media were used:

	MEDIUM 2	MEDIUM 3
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .....	2.0-2.5 grams	2.0 grams
K <sub>2</sub> HPO <sub>4</sub> .....	1.0 gram	1.0 gram
MgSO <sub>4</sub> ·7H <sub>2</sub> O .....	0.5 gram	0.5 gram
CaCl <sub>2</sub> .....	Trace	
NaCl .....		2.0 grams
FeSO <sub>4</sub> .....		0.4 gram
Distilled water .....	1000 cc.	1000 cc.
Magnesium carbonate (MgCO <sub>3</sub> ) .....	Excess (0.5 gram per flask)	Excess (0.5 gram per flask)

The medium is distributed into flasks (about 50 cc. portions), which are then plugged with cotton and sterilized at 15 pounds pressure for 15 minutes. It is best to sterilize the ammonium sulfate separately, as a 5 or 10 per cent solution, and then add it by means of a sterile pipette to the sterile flasks. Gibbs<sup>17</sup> employed medium 3, but reduced the ammonium salt to one gram and substituted a trace of ferric sulfate for the ferrous salt.

When 1 gram of soil is inoculated into the flasks containing the culture medium, growth will usually take place at 25° to 30°C. after 4 to 5 days, but sometimes only after weeks. Some soils, however, such as acid peat and certain acid forest soils,<sup>18</sup> may not contain the bacteria in question.<sup>19</sup> The organisms are not very sensitive to drying,<sup>17</sup> but the action of steam or volatile antiseptics is injurious and results in their destruction in

<sup>15</sup> Bonazzi, A. Jour. Bact. 4: 43-59. 1919.

<sup>16</sup> Winogradsky, 1904 (p. 64); Omeliansky, V. Centrbl. Bakt. II, 5: 537-549. 1899.

<sup>17</sup> Gibbs, M. W. Soil Sci. 8: 427-481. 1919.

<sup>18</sup> Migula, W. Centrbl. Bakt. II, 6: 365-370. 1900.

<sup>19</sup> See pp. 661, 669.

the soil. Manured and cultivated soils contain the nitrifying organisms in greatest abundance, especially in the upper layers of soil.

The appearance of growth is accompanied by the formation of nitrous acid and disappearance of ammonia. The former is demonstrated by the starch-zinc iodide reagent and the latter by Nessler's reagent. When all the ammonium is oxidized, a fresh portion of ammonium sulfate is added; a sterile 10 per cent solution of the salt is kept in a flask with a plugged graduated pipette: 1 cc. will be equivalent to addition of 0.2 per cent of the salt to the medium. When a second portion of the ammonium salt is added, it is oxidized much more rapidly since the specific organisms have already developed abundantly. A third portion is oxidized even more rapidly, until a certain limit of reaction is attained depending on the solution of the  $MgCO_3$  and the accumulation of nitrous acid. The culture is then transferred to a fresh flask with medium, using preferably a few drops from the bottom of the flask, since the bacteria form a sediment on the  $MgCO_3$ . Oxidation sets in now more rapidly and goes on more regularly. After four or five more transfers and repeated addition of ammonium sulfate to each culture before a new transfer is made, the culture is rich enough in the specific organisms and can be used for isolation purposes.

The nitrate-forming organism is present in crude culture together with the nitrite former and, even when the latter reaches its maximum, the former is still dormant. However, as soon as all the ammonia is transformed into nitrite, the nitrate former becomes active. When transfers are made from the crude culture, the stage of oxidation will account for the organism which will be prevalent. If the transfer is made at an early stage of oxidation, when the ammonium salt is still abundant, the nitrate former may be entirely eliminated in the process of several consecutive transfers. If nitrite is substituted in the medium, in place of the ammonium salt, and the culture is inoculated with soil or from a previous culture during the process of nitrate formation, and transfers are constantly made upon the nitrite medium, the nitrite forming organism may be entirely eliminated. The two bacteria can thus be separated by means of their characteristic metabolism.

*Solid media for the isolation and cultivation of the nitrite forming organism.* Silicic acid media were used first for the isolation of the nitrite forming bacterium.

Equal volumes of clear sodium or potassium silicate (specific gravity 1.05 to 1.06) and HCl (specific gravity 1.10) are mixed by pouring the first into the second; the mixture is then dialyzed in parchment paper dialyzers, for several days in

distilled water, which is repeatedly changed.<sup>20</sup> When the dialyzate gives no further reaction, other than mere turbidity, with  $\text{AgNO}_3$  the dialysis is completed. The clear solution contains about 2 per cent silicic acid and can be preserved for three months in clean glass-stoppered bottles and readily sterilized at  $115^\circ$  to  $120^\circ\text{C}$ . In addition to the silicic acid, four liquid solutions are prepared:

- |   |          |   |
|---|----------|---|
| 1. $(\text{NH}_4)_2\text{SO}_4$ .....           | 3 grams  | 2. 2 per cent solution of ferrous sulfate   |
| $\text{K}_2\text{HPO}_4$ .....                  | 1 gram   |   |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ..... | 0.5 gram | 3. Saturated $\text{NaCl}$ solution   |
| Distilled water.....                            | 100 cc.  | 4. Milk of magnesia, i.e., a thick suspension of finely powdered magnesium carbonate in distilled water |

Fifty cubic centimeters of the silicic acid solution are placed in a flask, then 2.5 cc. of solution 1 are added and 1 cc. of solution 2. Enough milk of magnesia is added to give the mixture a milky appearance (0.1 per cent sodium carbonate solution may be used in place of the magnesia). The mixture is then poured, with continued stirring, into sterile, small thin-walled Petri dishes. Finally, one drop of solution 3 is placed in the center of each plate. When allowed to rest in an horizontal position, the liquid solidifies in about an hour. To get a more solid medium, it is better to allow the dishes to rest 24 hours, then dry them out in the thermostat.

The medium is inoculated either directly into the flask before the plates are poured, or by placing a drop on the surface of the plate. The drop is spread over the surface of the medium with the tip of a sterile glass rod and the same rod is used for the inoculation of a second and third plate, so as to obtain a series of dilutions.

The dishes are incubated at  $25^\circ$  to  $30^\circ\text{C}$ . The development of an organism on the plate is first detected by chemical tests for the formation of nitrous acid and disappearance of ammonia. The tests are made by cutting pieces of the gel with a sterile loop and placing them in dishes containing the proper reagent. The culture can then be "fed" with 1 or 2 drops of a sterile 10 per cent ammonium sulfate solution to bring about further development of the organism.

The minute microscopic colonies are better studied on the clear (sodium carbonate) medium and soon after the addition of a fresh portion of ammonium sulfate. The colonies are at first colorless, then they become yellowish to brownish and finally dark brown; after a certain time (10 to 14 days), the dark colonies clear up, beginning from the edge toward the center, till finally the dark colony becomes colorless. A pure colony will show uniform fine granulation up to the edge, while contaminated colonies have an hyaline rim. A clear zone is formed

<sup>20</sup> Winogradsky, 1891 (p. 64); Omeliansky, 1899 (p. 66); another procedure for preparing silica gel is given on p. 191.

around each colony on the  $\text{MgCO}_3$  medium due to the fact that the latter is gradually dissolved by the nitrous acid; it is often difficult, however, to demonstrate this zone. The plate is carefully examined with a magnification of 50 to 100, and several clear surface colonies are selected for transfer. Winogradsky recommended the use of finely drawn sterile glass rods which are first dipped into the colony, then transferred into the flasks with sterile liquid medium and, by striking the bottom of the flask, the tip of the rod with the inoculum is broken off and left in the flask. A number of transfers are made, since, in many cases, the organism fails to develop. It is much more difficult to obtain a culture from the dark colonies (zooglea) than from the colorless colonies. To prove the purity of the culture, a few drops (about 0.5 cc.) of the liquid are inoculated into bouillon and meat peptone agar. No growth should take place after two weeks incubation; this combined with microscopic examinations indicates the absence of contaminations.

The silica gel can also be prepared by a modified procedure:<sup>21</sup> 24 grams of  $\text{K}_2\text{SiO}_3$  and 8.4 grams of  $\text{Na}_2\text{SiO}_3$  are dissolved in 500 cc. of water to give a concentration of 34.2732 grams of  $\text{H}_2\text{SiO}_3$  per liter. One-half this concentration of  $\text{H}_2\text{SiO}_3$  per liter gives a medium, which will solidify in approximately five minutes, thus making it suitable for plating. The mixture of the two salts lessens the danger of too great a concentration of sodium. The detrimental influence of too great a concentration of the sodium and potassium salts can still further be lessened by using a mixture of acids, such as equivalent solutions of  $\text{HCl}$ ,  $\text{H}_2\text{SO}_4$ , and  $\text{H}_3\text{PO}_4$ , thus giving in the finished medium chlorides, sulfates and phosphates of both bases.

The solutions of the three acids are standardized separately against the solution of the silicates, so that 1 cc. of each acid would just neutralize 1 cc. of the silicate solution, against methyl orange. Methyl red and brom cresol purple can also be employed. Before the final standardization, 0.5 gram  $\text{MgSO}_4$ , 0.01 gram  $\text{CaCO}_3$  or  $\text{CaO}$ , 0.01 gram  $\text{Fe}_2(\text{SO}_4)_3$  or  $\text{FeCl}_3$ , 0.01 gram  $\text{MnSO}_4$  and 1 gram of ammonium sulfate are added to the  $\text{HCl}$  solution.

The three acids are then mixed, taking 153.5 cc. of  $\text{HCl}$ , 77 cc. of  $\text{H}_2\text{SO}_4$  and 116 cc. of  $\text{H}_3\text{PO}_4$ . One cubic centimeter of this mixture will just neutralize 1 cc. of the silicate solution, using phenolphthalein as an indicator. The two solutions are placed in sterile bottles connected by siphons with automatic burettes, the overflow caps of which are plugged with cotton to prevent contamination from the air. The solutions are allowed to stand several hours, to sterilize the containers, then 5 cc. portions of the acid mixture followed by the same amounts of silicate mixture are placed in sterile Petri dishes, which are then rotated thoroughly; the jelly sets in five minutes. This medium has, however, never been tested out sufficiently; it is possible that the osmotic concentration of the salts may prove excessive.

<sup>21</sup> Stevens, F. L. and Temple, J. C. *Centrbl. Bakt.* II, 21: 84-87. 1908; Doryland, C. J. T. *Jour. Bact.* 1: 135-152. 1916.

In addition to the silica plate method, three more methods are available for the isolation of the nitrite forming bacteria.

*Method 1.* Agar may be prepared according to Beijerinck,<sup>22</sup> whereby 3 per cent agar is dissolved in distilled water, filtered and allowed to solidify in thin layers in glass containers. The solidified agar is then cut up into pieces, covered with distilled water and incubated at 37° for two weeks; the soluble constituents of the agar come into solution and are decomposed; the water is changed several times. The agar is so purified that it can be used for the cultivation of the organisms. It is preserved either under water or in a dried condition. Two-tenths per cent  $\text{NH}_4\text{NaHPO}_4 \cdot 4\text{H}_2\text{O}$  and 0.05 per cent KCl and chalk are then added to the agar and brought into solution. The plates have a milky appearance. The agar may be washed in distilled water for several days, then dried at 60°C. A 2.5 per cent solution of agar is then made, tubed in 10 cc. portions and sterilized in the autoclave at 15 pounds pressure. Three solutions are then prepared and sterilized in small portions.

	grams in 100 cc.
1. $\text{K}_2\text{HPO}_4$ .....	1.5
2. $(\text{NH}_4)_2\text{SO}_4$ .....	1.5
$\text{MgSO}_4$ .....	0.75
$\text{Fe}_2(\text{SO}_4)_3$ .....	0.02
3. $\text{NaCl}$ .....	3.0
$\text{Na}_2\text{CO}_3$ .....	1.5

The agar is melted and cooled to 40°C. Portions (1 cc.) of the three solutions are placed in sterile Petri-dishes, inoculum is added, then the melted agar. All the contents of the plate are properly mixed. If it is desired to use  $\text{MgCO}_3$ , it should be added to the plate directly, at the time of pouring, and  $\text{Na}_2\text{CO}_3$  omitted from solution 3. The colonies of the bacterium develop on this medium only slowly and a prolonged incubation period (3 to 4 weeks) is required. Agar media are also more favorable to the development of contaminating organisms than silica gel.

*Method 2.* This is the magnesium carbonate—gypsum block method.<sup>23</sup> A mixture of 300 grams gypsum ( $\text{CaSO}_4 \cdot \text{H}_2\text{O}$ ), 30 grams  $\text{MgCO}_3$  and 3 grams  $\text{MgNH}_4\text{PO}_4$  is made into a homogeneous putty-like mass by means of water or water extract of a fertile soil (using 250 grams of soil per liter of water), mixing 8 parts of the powder and 3 parts of the liquid. The paste is then put upon a glass plate and, by means of a knife, streaked out to a thickness of 0.5 to 0.75 cm. Round portions are cut out for the plates and oblong for the tubes. After the material has completely solidified, it is sterilized together with the glass containers. A small amount of the sterile nutrient liquid, without the ammonium

<sup>22</sup> Beijerinck, W. M. Centrbl. Bakt. I, 19: 257-267. 1896.

<sup>23</sup> Omeliansky, W. L. Centrbl. Bakt. II, 5: 652-655. 1899; Makrinov, J. Centrbl. Bakt., II, 24: 415-423. 1910.

sulfate and  $\text{MgCO}_3$ , is placed at the bottom of the container, and the surface of the plate is inoculated from the liquid culture. The nitrite forming bacteria develop on these blocks as yellow-brown colonies.  $\text{MgCO}_3$  alone can be used, to which the nutrient solution is added. The yellowish colonies sink into this medium due to the dissolution of the  $\text{MgCO}_3$  by the nitrous acid. The use of filter paper, partly covered with the nutrient solution, in addition to some  $\text{MgCO}_3$ , has also been suggested.<sup>24</sup> The organism forms minute yellow dots becoming gradually brown. However, even the most recent students on this subject<sup>25</sup> found that the original silica gel method of Winogradsky is still the best for the isolation of the nitrite forming bacteria.

*Method 3.* Winogradsky recently suggested the possibility of using a direct method for the isolation of nitrite forming, as well as other bacteria. This new method promises to replace all the other methods, due to the rapidity with which the organisms can be isolated. A series of plates containing silica gel, prepared by one of the methods outlined above, and ammonium salt as the sole source of energy are inoculated by placing minute particles of soil into the gel all over the plates; these are then incubated. The nitrite-forming bacteria develop in the form of a zooglea-like zone around the soil particles, and can be readily isolated by transferring to sterile liquid medium.

*Morphology of the nitrite forming bacterium.* In the process of nitrification we are dealing not with one organism, but with a group of closely related organisms. One strain was isolated<sup>26</sup> from soils of Western Europe and was called *Nitrosomonas* (*Nitr. europea* Win.). Another strain was isolated from soils of South America and Australia and was called *Nitrosococcus*.

When a vigorous culture of *Nitrosomonas* is inoculated into the sterile liquid medium, an appreciable nitrite reaction is obtained in 2 to 3 days, reaching a maximum in 5 to 6 days. When the culture is examined microscopically, very few organisms are found in the supernatant liquid, but rare, compact, variable (10 to  $50\mu$ ) zooglea recognized with difficulty are formed in the sediment. When a drop of KI-I solution is added, the cells are easily recognized. In 8 to 10 days, the liquid becomes opalescent and, on examination in a hanging drop, it is found to consist of swarming, ellipsoidal, motile microbes, as seen in Plate II. This shows the zooglea broken up into a swarm stage.

The cells of the *Nitr. europea* are always oblong, similar to a zero, never coccus-like, 1.2 to  $1.8\mu$  long by 0.9 to  $1\mu$  wide. They can be stained with all ordinary basic aniline dyes. The motile cells of the swarm carry on one end a moderately long flagellum; the latter can be stained

<sup>24</sup> Omeliansky, W. L. Centrbl. Bakt., II, 8: 785-787. 1902.

<sup>25</sup> Bonazzi, A. Bot. Gaz. 68: 194-207. 1919.

<sup>26</sup> Winogradsky, 1892 (p. 64).



by the Loeffler method, by adding 10 to 15 drops of 1 per cent sodium carbonate solution to the ferrotannate, or by the method of Zettnow.

In some cases the motile stage may be predominant over the zooglea formation or vice versa. It is important to note that the predominance of the particular stage is characteristic of the strain, so that one might suspect that we are dealing here with two distinct races. The two stages are also distinguished by their rapidity of oxidation of ammonia, the motile stage being the stronger. Winogradsky suggested that the cause for this lies in the fact that the active stage (monas) consumes more energy and comes more readily in contact with the ammonia and oxygen than the non-motile zooglea. The zooglea are probably resting stages, being also more resistant to drying. Gibbs isolated the *Nitrosomonas* from soils of North America and found it to be 1.2 to 1.5 by 0.9 to 1.0 $\mu$  in size, rounded or oval, which stained uniformly. The organism was found chiefly in the free cell stage. Thermal death point of the organism was between 53° and 55°C. (10 minutes).

Other organisms also capable of oxidizing ammonia to nitrite, but having different morphological characters, were isolated by Winogradsky from soils in Europe. The form isolated from a soil of St. Petersburg was a true coccus, about 1 $\mu$  in diameter, sometimes forming zooglea and sometimes growing free, but never in the swarm stage. A constant property of its morphology is a central nucleus-like body, made visible by various stains, particularly by methylene blue.

The *Nitr. javanensis* is a still smaller coccus (0.5 to 0.6 $\mu$ ) and has been isolated by Winogradsky from the soil of Buitenzorg, Java. This form

## PLATE II

### NITRIFYING BACTERIA

7. Surface colonies of *Nitrosomonas* on silicic acid gel, stained with carbol fuchsin,  $\times 130$  (from Gibbs).
8. Surface colony of *Nitrosomonas* on silicic acid gel, stained with carbol fuchsin,  $\times 800$  (from Gibbs).
9. *Nitrosomonas europea*,  $\times 660$  (from Winogradsky).
10. *Nitrosomonas javanensis*,  $\times 660$  (from Winogradsky).
11. Colonies of *Nitrobacter*; deep-seated colonies on washed agar, unstained,  $\times 240$  (from Gibbs).
12. *Nitrobacter* from culture in liquid medium; stained with carbol fuchsin,  $\times 1660$  (from Gibbs).
13. *Nitrobacter* from nitrite-agar cultures, 2 months old (from Fred and Davenport).
14. *Nitrobacter* from nitrite-agar cultures, 15 days old, showing polar flagella (from Fred and Davenport).



7



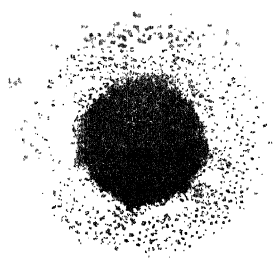
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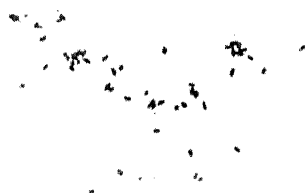
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14



grows both in the monas and zooglea stages, the first having very long (up to  $30\mu$ ) flagella. Free cells are present only in the swarm stage, mostly in pairs. Winogradsky also isolated from the soil of Quito, South America, a non-motile coccus (*Nitrosococcus*) termed by Migula *Micr. nitrosus*. This organism consists of large cocci  $1.4$  to  $1.7\mu$  in diameter, always growing as free cells and never forming zooglea, while the motile stage could never be demonstrated. It is obligate aerobic, and forms rather large, opaque yellowish colonies on the silica gel but the colonies are made up of free cells. The cells appear larger in the living state than in the stained preparation, probably due to a thick gelatinous membrane which does not stain or becomes invisible on desiccation. Bonazzi<sup>27</sup> isolated the *Nitrosococcus* also from the soil of North America: it forms small yellowish colonies ( $224$  by  $160\mu$  on the silica gel) surrounded by a colorless halo, due to the solution of the  $MgCO_3$ . Colonies of  $1$  mm. in diameter have been obtained by renewing the  $(NH_4)_2SO_4$  in the plates when necessary. The organism was found to be present in two stages: (1) as large cocci,  $1.25\mu$  in diameter, of a slightly irregular form, occurring in thick gelatinous masses; when the cultures are in the process of active oxidation, the large cocci give rise to small cocci; (2) a form which leaves the gelatinous mass and become free. The hanging drop cultures showed no motility.

The organism is stained as follows. The cover glass preparation is fixed in flame, treated with mordant  $1$  minute in the cold with a  $0.25$  per cent solution of malachite green in distilled water, washed with cold water, and stained cold with a  $0.25$  per cent water solution of gentian violet for another minute. The stain is then rapidly washed with water previously heated to  $50$  to  $60^\circ C$ . The megalococci are stained deep purple and the small cocci purple-black.

The existence of the several forms of nitrite-forming organisms in the soils from different continents was explained by Winogradsky as due to the probability that local conditions favored the adaptation of a particular variety. Recently,<sup>27a</sup> Winogradsky suggested to classify the nitrite-forming bacteria into 3 groups: 1. Free, motile forms, present in the soil as rods and cocci—*Nitrosomonas*. 2. Zooglea, composed of cocci united in rounded masses and surrounded by a membrane—*Nitrocystis*. 3. Spiral shaped forms—*Nitrospira*. The various forms occur in different soils, the last found only in uncultivated soils.

<sup>27</sup> Bonazzi, 1919 (p. 71).

<sup>27a</sup> Winogradsky, S. Compt. Rend. Acad. Sci. **192**: 1000-1004. 1931.

*Nitrate forming bacteria (Nitrobacter).* The organism that is able to oxidize nitrite to nitrate was discovered by Winogradsky in 1891 in the solutions where nitrate formation was taking place.

It was finally cultivated in a sodium nitrite medium to which the ordinary nutrients and magnesium carbonate or sodium carbonate have been added as follows:

NaNO <sub>2</sub> .....	1.0 gram	NaCl.....	0.5 gram
K <sub>2</sub> HPO <sub>4</sub> .....	0.5 gram	Na <sub>2</sub> CO <sub>3</sub> (anhydride)...	1.0 gram
MgSO <sub>4</sub> .....	0.3 gram	FeSO <sub>4</sub> .....	0.4 gram
		Distilled water.....	1000 cc.

(The ferrous sulfate may be reduced to a trace and the anhydrous Na<sub>2</sub>CO<sub>3</sub> to 0.5 gram.)

Fifty cubic centimeter portions of the sterile solution in flasks are inoculated with soil. The course of the reaction is followed by the disappearance of the nitrous acid and the appearance of nitric acid (using diphenylamine and concentrated sulfuric acid). When all the nitrite has been oxidized, fresh portions of the salt are added, in the form of a sterile solution as in the case of the ammonium sulfate, and the culture is studied microscopically, using carbol fuchsin for staining.

The inoculated solutions show no turbidity or pellicle formation. It is only after repeated additions of nitrite that a bluish slime can be distinguished on the bottom and wall of the flask wherever it is in contact with liquid. When this slime is examined microscopically, it is found to consist of a layer of minute, spindle-shaped (generally) rods staining with difficulty. After several transfers into fresh flasks containing sterile liquid medium, the culture is sufficiently enriched, so that plates can be prepared.

The following agar medium is used for the isolation and cultivation of the organism:

NaNO <sub>2</sub> .....	2.0 grams	Agar.....	15.0 grams
Na <sub>2</sub> CO <sub>3</sub> (anhydride)...	1.0 gram	Tap water.....	1000 cc.
K <sub>2</sub> HPO <sub>4</sub> .....	0.5 gram		

The surface of the solidified agar plate is smeared with a drop of the solution in which the organism has developed, and the plates are allowed to incubate 14 days at 30°. The streak then appears opaque and numerous small rounded drop-lets are differentiated with the naked eye. The sub-surface colonies are shining, slightly brownish, of various shapes developing in two weeks to a diameter of 30 to 50 $\mu$ . On the surface of the plate, the colonies appear as round homogeneous drops, reaching, in two weeks, a diameter of 100 to 180 $\mu$ . On slants the growth is dirty white, with a large, semi-fluid drop at the bottom. When a loop of this material is transferred into a 25 cc. portion of nitrite solution, the nitrite reaction disappears in 3 to 4 days.

Care should be taken not to mistake other bacteria for the nitrate former, since several soil bacteria grow on this medium. The smallest colonies are selected and carefully checked up with the disappearance of the nitrite reaction, combined with prolonged incubation (3 weeks at 30°C.). Transfer is then made of characteristic colonies by means of the open capillary glass rods described above. From a series of transfers, some will be found to develop into pure cultures. To ascertain the purity of the culture, several drops of the liquid culture are added to bouillon or agar.

*Nitrobacter* is a non-motile, rod-shaped bacterium, obligate aerobic, non-spore forming. The cells are stained with carbol fuchsin, and can then be washed with dilute acidified alcohol. It is 1 by 0.3 to 0.4 $\mu$  in size, with one or both ends pointed; the staining is not uniform, the central part being stained, while the pointed ends remain almost colorless. In Loeffler's alkaline methylene blue, only the nucleus-like bodies are stained well, but not the surrounding cell. By staining in warm gentian-violet, then washing with a 2 per cent NaCl solution, the cells are found to be 1.2 to 1.5 by 0.6 to 0.7 $\mu$ , surrounded by a capsule, commonly found in single cells or in pairs. The thermal death-point is 56° to 58°C. The colonies on agar are rounded and light-brown, in 7 to 10 days at 28°C. After two weeks, they are darker in color; deep colonies are 30 to 50 $\mu$  in diameter and surface colonies are 50 to 150 $\mu$  in diameter, with a tendency to spread. The nitrate bacteria may also be isolated directly from the silica gel plate, when nitrite is used as the only source of energy.

Beijerinck<sup>28</sup> claimed that the nitrate-forming bacterium can grow in the presence of various organic substances but that the organism loses some of its power of oxidation after growing in the presence of soluble organic matter. Accordingly, he suggested that growth and oxidation are two distinct functions and that even if small amounts of organic substances (0.05 per cent) did not prevent the oxidation of nitrite to nitrate, reproduction of the organism in solutions containing large amounts of these substances caused a stable modification in their physiology. Fred and Davenport<sup>29</sup> obtained no evidence to support the statements of Beijerinck. Contrary to the general opinion, they found that certain forms of organic matter benefit rather than injure the nitrate-forming bacterium. They grew the *Nitrobacter* on washed nitrite agar and on slants of Nährstoff-Heyden agar with or without

<sup>28</sup> Beijerinck, M. W. *Folia Microb.* 3: 91-113. 1914.

<sup>29</sup> Fred, E. B. and Davenport, A. *Soil Sci.* 11: 389-407. 1921.

nitrite. The organism does not reproduce in Nährstoff-Heyden solution, which is non-toxic, while beef-infusion and peptone-beef infusion, in higher concentrations, are toxic. The harmful material is non-volatile and can be removed by extraction with ether or alcohol. Nitrobacter will live 2 to 6 weeks in 1 per cent solutions of gelatin, peptone, casein, yeast water and milk, and also in distilled water without any further development. These media do not decrease the oxidation of nitrite by the organism; asparagine,  $(\text{NH}_4)_2\text{SO}_4$ , and urea retard the oxidation; sealed agar slants of Nitrobacter were kept more than a year without serious injury to their power of oxidation.

Winogradsky<sup>30</sup> definitely pointed out that growth and nitrite oxidation are inseparable functions; organic matter (1 per cent peptone) may paralyze the organism, but does not kill it and does not change it, since, when transferred upon proper media, it resumes its activities.

*Occurrence of nitrifying bacteria in the soil.* All soils, not very acid in reaction, contain bacteria capable of oxidizing ammonium salts to nitrites and the latter to nitrates. The limiting acidity for the development of these bacteria in the soil is pH 4.0 to 3.7, while the optimum reaction is at pH 6.8 to 7.3.<sup>31</sup> When a soil more acid in reaction than the minimum for their development is treated with lime, the organisms will gradually appear in the soil; however, inoculation with a good fertile soil is often practicable, so as to introduce the organisms immediately. The nitrifying bacteria are not killed when soils are dried at ordinary temperatures; hence they can be distributed by dust. The numbers of the nitrifying bacteria per gram of soil vary from a few to 24,000. The method commonly used for this determination consists in diluting the soil with sterile water, then adding 1 cc. portions of the various dilutions to the proper media. Positive growth indicates a minimum number of organisms. It is possible, however, that many cells have to be added to a liquid medium, before growth can take place, since conditions are not made as favorable for their development in artificial liquid media as in normal soil. In humid soils, the bacteria are present in the upper few inches and rapidly disappear in the subsoil. However, in arid soils, they occur to a depth of many feet.

In addition to the typical nitrite and nitrate bacteria described above, various isolations have been made of other organisms capable of producing nitrite and nitrate. These range from typical autotrophic

<sup>30</sup> Winogradsky, S. Compt. Rend. Acad. Sci. 175: 301-303. 1922.

<sup>31</sup> Gaarder, T., and Hagem, O. Bergens Museum Aarbook. 1922-3, No. 1; Meddel. No. 11, Vestland. Forst. Versoksta. Bergen. 1928.

bacteria, like the *Nitromicrobium* of Stutzer and Hartleb,<sup>32</sup> to forms possessing properties altogether uncharacteristic of autotrophic organisms, such as cellulose decomposition, gelatin liquefaction and nitrate reduction;<sup>33</sup> these organisms comprise not only bacteria but also actinomycetes. It still remains to be established whether the nitroso reaction obtained in these cultures (mostly organic media) is due to the presence of nitrites produced by oxidation of the ammonium-ion, or to the formation of nitrites by reduction of traces of nitrates in medium or to the presence of other substances giving the nitroso reaction. In all these studies, no attempt has been made to study the energy utilization by the organisms. Even if it becomes definitely established that certain heterotrophic soil organisms are capable of producing small quantities of nitrite by oxidation, this process need not be confused with the organisms of Winogradsky, capable of utilizing the energy obtained in the oxidation of nitrogen compounds for the chemosynthetic assimilation of carbon dioxide. The earlier ideas of Winogradsky have been again confirmed recently.<sup>34</sup>

*Bacteria deriving their energy from the oxidation of sulfur and its compounds.* The sulfur bacteria do not form any uniform group of microorganisms, as in the case of the nitrifying bacteria, either morphologically or physiologically. Morphologically they are found among the Desmobacteriaceae and among the Bacteriaceae. Physiologically they may oxidize hydrogen sulfide and other sulfides, elementary sulfur, or thiosulfate and they may act either in an acid or in an alkaline reaction. Some are obligate autotrophic and some are facultative. The bacteria which are found in normal, fertile soils or those that become active in the soil, when introduced, are limited chiefly to the genus *Thiobacillus* among the Bacteriaceae.

All microorganisms require minute quantities of sulfur for the synthe-

<sup>32</sup> Stutzer, A. and Hartleb, R. Mitt. landw. Inst. Breslau, 1: 75-99, 197-232. 1901.

<sup>33</sup> Sack, J. Centrbl. Bakt., II, 62: 15-24. 1924; 64: 32-37, 37-39. 1925; Mischustin, E. H., Viestnik Bact. Agr. Sta. 25: 28-39; Runov, E. B. Ibid. 40-57; Nelson, D. H. Iowa St. Coll. Jour. Sci. 3: 113-175. 1929; Cutler, D. W. Nature, 125: 168. 1930; Cutler, D. W. and Mukerji, B. K. Proc. Roy. Soc. B. 108: 384-394. 1931.

<sup>34</sup> Heubült, J. Planta, Arch. wiss. Bot. 8: 398-422. 1929; Engel, H. Ibid., 423-426; Engel, H. Arch. Mikrob. 1: 445-463. 1930; Rubentschik, L. Centrbl. Bakt. II, 77: 1-18. 1929; Winogradsky, S. Bull. Inst. Past. 25: 305-306; 28: 681-687. 1930; Nelson, D. H. Science 71: 541-542; Centrbl. Bakt. II, 83: 280-311. 1931.



sis of their protoplasm. Various bacteria and even some fungi seem to be capable of oxidizing small amounts of sulfur. But few bacteria work over much larger quantities of sulfur than are necessary for their body structure, since they utilize the sulfur or its compounds as a source of energy. The sulfur is to the sulfur bacteria, as the ammonium sulfate is to the *Nitrosomonas* and *Nitrosococcus*, the nitrous acid and nitrite to *Nitrobacter* and the carbon compounds to the heterotrophic bacteria.

The sulfur bacteria, or those bacteria which are capable of obtaining the energy necessary for their growth from the oxidation of sulfur or its compounds, should be distinguished from other bacteria taking part in the sulfur cycle, such as those liberating  $H_2S$  in the hydrolysis of proteins or in the reduction of sulfates.

COLORLESS, THREAD-FORMING SULFUR BACTERIA, ACCUMULATING SULFUR WITHIN THEIR CELLS (Nos. 15-18, Pl. III). The representatives of this group are able to act only upon  $H_2S$  in the medium. In view of the fact that these are primarily water and mud forms, only the general principles involved are discussed. This group of sulfur oxidizing bacteria consists of three genera: *Beggiatoa*, including motile organisms forming

### PLATE III

#### SULFUR AND IRON BACTERIA

15. *Beggiatoa alba*, thread forming sulfur-oxidizing bacterium: a, in liquid culture rich in  $H_2S$ ; b, culture kept 24 hours in liquid freed from  $H_2S$ ; c, 48 hours later in the same liquid (sulfur droplets have disappeared, cell division takes place and protoplasmic contents are left),  $\times 600$  (after Omeliansky).

16. Thread-forming, sulfur-oxidizing bacteria: (x) *Beggiatoa media* and (y) *Beggiatoa minima*,  $\times 600$  (after Omeliansky).

17. *Thioploca ingrica*,  $\times 200$  (after Wislouch and Omeliansky).

18. Young threads of *Thiothrix nivea*,  $\times 600$  (after Omeliansky).

19. *Thiophysa macrophysa*, showing drops of sulfur on periphery and oxalate crystals in center,  $\times 660$  (after Nadson and Omeliansky).

20. *Thiospirillum winogradskii*: a  $\times 100$  and b  $\times 660$  (from Omeliansky).

21. *Chromatium okénii*,  $\times 660$  (after Omeliansky).

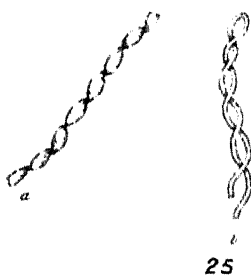
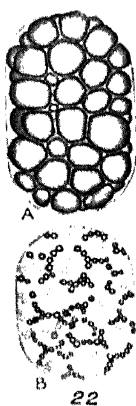
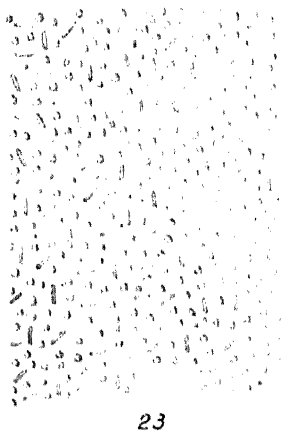
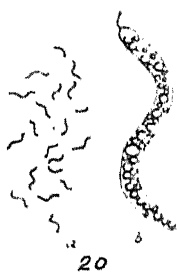
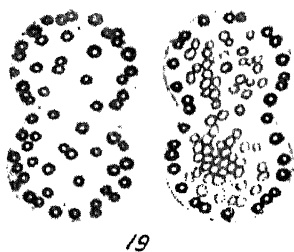
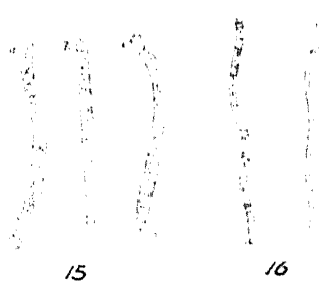
22. *Achromatium oxaliferum*: A, showing the calcium bodies, but not sulfur; B, without the calcium bodies, but with a number of droplets of sulfur (from Nadson and Wislouch).

23. *Thiobacillus thioparus*, showing drops of precipitated sulfur among the rod-shaped organisms,  $\times 1000$  (from Dügge).

24. *Thiobacillus thiooxidans*,  $\times 660$  (Original).

25. Diagrammatic sketch of several typical iron bacteria: a, *Spirophyllum ferrugineum*; b, *Gallionella ferruginea*; c, *Leptothrix ochracea*,  $\times$  about 720 (from Harder, by courtesy of U. S. Geological Survey).

26. *Cladothrix dichotoma*,  $\times 190$  (after Molish).





no sheaths; *Thiothrix*, fastened forms forming no sheaths; and *Thioploca*, thread-forming bacteria, surrounded with a jelly-like sheath. The Beggiatoa was the first organism to attract attention as having to do with the oxidation of sulfur or its derivatives. Cramer<sup>35</sup> pointed out that the granules found within the cells of Beggiatoa consisted of sulfur. Cohn<sup>36</sup> then proposed the theory that the Beggiatoa and the purple bacteria produce hydrogen sulfide by the reduction of sulfates. But it was Winogradsky<sup>37</sup> who demonstrated that the hydrogen sulfide is produced by other bacteria and is oxidized by the Beggiatoa to sulfur and sulfuric acid.

This oxidation is so important for the very existence of these organisms that, when the hydrogen sulfide is taken out of the medium, they oxidize the sulfur present within their cells and, when this is used up, they die out. The energy liberated in this process is utilized by the organisms for the assimilation of carbon dioxide. For every gram of carbon, 8 to 19 grams of sulfur are consumed. If there is enough  $H_2S$ , the presence of traces of organic substances and nitrates in the water is sufficient for the development of these organisms, while the presence of sugars, peptone and like nutrients will stimulate the growth of other microbes but will injure these sulfur bacteria.

According to Winogradsky, the sulfuric acid formed is neutralized by the calcium carbonate or bicarbonate present in the water, since the reaction of the water cultures of these bacteria was not found to become acid. In reference to the physiology of these organisms, the results of Winogradsky can be summarized as follows: (1) The sulfur bacteria oxidize hydrogen sulfide and accumulate sulfur in the form of small spheres, consisting of soft amorphous sulfur which never crystallizes in the living cells. (2) They oxidize the sulfur to sulfuric acid, which is at once neutralized, by the carbonates present, into sulfates. (3) Without sulfur, the organisms soon die off. (4) They can live and multiply in liquid containing only traces of organic substances.

Keil<sup>38</sup> claimed to have isolated pure cultures of Beggiatoa and Thiothrix, and found that these organisms are capable of living in media free from any traces of organic matter, although the presence of small quan-

<sup>35</sup> Cramer, In Müller, C., Chemisch-physikalische Beschreibung der Thermen, von Boden in der Schweiz. 1870.

<sup>36</sup> Cohn, F. Beitr. Biol. Pflanz. H. 3: 141-207. 1875.

<sup>37</sup> Winogradsky, S. Beiträge zur Morphologie und Physiologie der Bakterien. I. Schwefelbakterien. Leipzig. 1883; Ann. Inst. Past. 3: 1883; Bot. Ztg. 45: 489, 513, 529, 545, 569, 585, 606. 1887.

<sup>38</sup> Keil, F. Beitr. Biol. Pflanz, II: 335-372. 1912.

tities of organic substances is not detrimental to them. The raw cultures were obtained by Keil by placing a layer of black mud containing these bacteria on the bottom of a glass container, 3 to 4 cm. high, covering it with 2 to 3 cm. of river water and placing in the dark, at room temperature. The Beggiatoa formed a white layer over the mud. The Thiothrix could be easily distinguished by the fact that they were fastened at one end. By adding water from a sulfur spring to Petri dishes, then placing these under a bell-jar, the amount of gas necessary for the growth could readily be ascertained. Ammonium salts were found to be used as sources of nitrogen and only carbonic acid as a source of carbon. Carbon dioxide pressure may vary within the limits of 0.5 and 350 mm. (25 mm. is the optimum); oxygen may vary within 10 to 20 mm., and  $H_2S$  within 0.6 to 1.7 mm. The presence of carbonates is important for the neutralization of the acids.

The pure cultures were obtained from the enriched culture by the mere mechanical process of washing out all other organisms first with ordinary water and then with sterile water. This was followed by growth under the bell-jar, at definite gas pressures and frequent changes of medium. Further information on this group of organisms is found in the work of Omeliansky,<sup>39</sup> Düggei,<sup>40</sup> Bavendamm,<sup>41</sup> and others.<sup>42</sup> The Thioploca has been studied in detail by Wislouch<sup>43</sup> and Kolkwitz.<sup>44</sup>

THE COLORLESS ORGANISMS NOT FORMING THREADS AND CONTAINING SULFUR WITHIN THEIR CELLS, ALSO ACTING ONLY ON  $H_2S$  IN THE MEDIUM (Nos. 19-22, Pl. III). This group includes organisms of various forms. They may be obtained by placing the cut rhizomes of water plants, together with the mud, into tall glass cylinders with some river or canal water and a few grams of calcium sulfate. When placed in the dark,  $H_2S$  will be produced in 5 to 10 days, and the colorless non-thread-forming sulfur bacteria are found in 3 to 6 weeks. The nature of the plant or animal material, nature of the mud and quantity of  $H_2S$  produced, determine which species will predominate. Among the organisms described at various times we might mention: *Monas mülleri* and *Monas fallax*,<sup>42</sup> *Thiophysa volutans*,<sup>45</sup> *Thiospirillum winogradskii*,<sup>46</sup>

<sup>39</sup> Omeliansky, W. L. Lafar's Handb. techn. Mykol. 3: 214-244. 1904.

<sup>40</sup> Düggei, M. Neujahrsbl. Naturf. Gesell. Zürich. 1919, No. 121, 43 p.

<sup>41</sup> Bavendamm, W. G. Fischer. Jena. 1924.

<sup>42</sup> Hinze, G. Ber. deut. bot. Gesell. 31: 189-202. 1913. Molisch, H. Centrbl. Bakt. II, 33: 55-62. 1912.

<sup>43</sup> Wislouch, S. M. Ber. deut. bot. Gesell. 30: 470-473. 1912.

<sup>44</sup> Kolkwitz, R. Ber. deut. bot. Gesell. 30: 662-666. 1912.

<sup>45</sup> Hinze, G. Ber. deut. bot. Gesell. 21: 309-316. 1903.

<sup>46</sup> Omeliansky, W. L. Centrbl. Bakt. II, 14: 769-772. 1905.

*Thiovulum*,<sup>45</sup> *Spirillum*, *Bacterium bovista* (2 to 4 by 0.6 by 1.5 $\mu$ ), *Bacillus thiogenes* (2 to 6 by 0.9 to 1.34 $\mu$ ), and *Achromatium*.<sup>47</sup> Most organisms belonging to this group have been found in water and in mud; few of them have been obtained in pure cultures. They play an important part in the formation of the curative muds.<sup>48</sup> According to Nadson, some of these organisms, like *Achromatium* and *Thiophysa*, can accumulate in their cells sulfur as well as oxalate crystals. Jegunow described two sulfur bacteria: *Thiobacterium*  $\alpha$ , a motile, colorless, slightly curved organism, 4.5 to 9 $\mu$  long and 1.4 to 2.3 $\mu$  wide, containing a finely granulated plasma and large sulfur granules, and *Thiobacterium*  $\beta$ , motile, colorless, curved, 2.5 to 5 by 0.6 to 0.8 $\mu$ , and containing a row of shining sulfur granules. Various bacteria belonging to this group have been reported<sup>49</sup> to occur in the soil, namely: *Spirillum agilissimum* filled with black sulfur granules, measuring about 6 to 10 by 1.8 to 2.0 $\mu$ , having rapid motility, and isolated from river mud in Gratz; *Chromatium cuculliferum* which is round to slightly elliptical, 6 by 4 $\mu$ , of a slow motility, with black, shining, sulfur drops always found in one pole, with one flagellum on the granule-free pole. This latter form was found in rotting mass of algae in the garden basin at Gratz. However, since none of the forms has been considered from the point of view of its rôle in soil transformations, their importance in the soil is doubtful. A detailed study of the morphology and biology of *Achromatium oxaliferum* Schew., containing granules of a calcium salt (oxalate, carbonate or thiosulfate) and sulfur has been made by Nadson and Wislouch.<sup>50</sup>

The red or purple sulfur bacteria are distinguished from the bacteria described above by the production of a red, red violet or red brown pigment which is unevenly distributed throughout the cell; in addition to the red pigment (bacterio-purpurin), there is also present in all these bacteria a green pigment (bacterio-chlorin). These bacteria are found abundantly in sulfur springs and in mud waters. Not all the pigmented bacteria are able to utilize hydrogen sulfide and not all of them

<sup>47</sup> Nadson, G. A. Bull. Jard. Bot. St. Petersburg, 13: 106-112. 1913; Jour. Microb. (Russian) 1: 52-72. 1914; West, G. S. and Griffith, B. M. Ann. Bot. 27: 83-91. 1913.

<sup>48</sup> Jegunow, M. Centrbl. Bakt. II, 2: 11-21, 441-449; 478-482; 739-752. 1896.

<sup>49</sup> Gicklehorn, J. Centrbl. Bakt. II, 50: 415-427. 1920.

<sup>50</sup> Nadson, G. A. and Wislouch, C. M. Bull. Jard. Bot. Rep. Russe. 22: 1-24. 1923.

accumulate sulfur within their cells. Sulfur plays an important rôle<sup>51</sup> in the metabolism of those purple bacteria which are capable of using hydrogen sulfide, thus confirming the earlier ideas of Winogradsky.

COLORLESS BACTERIA THAT DO NOT ACCUMULATE SULFUR WITHIN THEIR CELLS, BUT PRODUCE SULFUR ABUNDANTLY FROM THIOSULFATE AND HYDROGEN SULFIDE OUTSIDE OF THEIR CELLS. A few of the bacteria belonging to this group oxidize thiosulfate without the precipitation of sulfur (No. 23, Pl. III). These organisms were first demonstrated by Nathanson<sup>52</sup> (1902) in sea water. They were found to be able, by means of oxidation of hydrogen sulfide or sodium thiosulfate, to reduce carbonic acid and construct organic substances from it. Nathanson used a medium of the following composition:

Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> .....	2-10 grams	NaCl.....	30.0 grams
KNO <sub>3</sub> .....	1 gram	MgCO <sub>3</sub> .....	some
Na <sub>2</sub> HPO <sub>4</sub> .....	0.5 gram	Water.....	1000 cc.
MgCl <sub>2</sub> .....	2.5 grams		

A good growth of these bacteria was obtained after 1 to 2 days, in the form of a white pellicle covering the surface; this consisted of rod-shaped organisms intermixed with amorphous sulfur.

On adding agar to the above medium Nathanson has been able to isolate the organism in pure culture. In the absence of the carbonate, but in the presence of air containing carbon dioxide, the growth was much slower. In the absence of both carbonate and carbon dioxide, no growth took place, even in the presence of various organic substances. The medium did not become acid even in the absence of carbonate. While no sulfur accumulated within the cell, there was an abundant production of free sulfur outside of the cell, not in direct contact with the colony but at some distance from it. This led to the theory of extra-cellular oxidation. Nathanson suggested that the sulfur is produced in a secondary reaction between the undecomposed thiosulfate and the tetrathionate formed from the oxidation of the thiosulfate.

Beijerinck<sup>53</sup> employed the following medium:

Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> ·5H <sub>2</sub> O.....	5.0 grams	NH <sub>4</sub> Cl.....	0.1 gram
NaHCO <sub>3</sub> .....	1.0 gram	MgCl <sub>2</sub> .....	0.1 gram
Na <sub>2</sub> HPO <sub>4</sub> .....	0.2 gram	Water.....	1000 cc.

<sup>51</sup> Bavendamm, W. Die farblosen und roten Schwefelbakterien. G. Fischer, Jena. 1924. See also Molisch, H. The Purpurbakterien nach neuen Untersuchungen. G. Fischer. 1907.

<sup>52</sup> Nathanson, A. Mitt. Zool. Station, Neapel, 15: 655. 1902.

<sup>53</sup> Beijerinck, M. W. Centrbl. Bakt. II, 11: 593-599. 1904.

The medium was left unsterilized and was inoculated with canal water and incubated at 28° to 30°C. In 2 to 3 days, the surface of the medium became covered with free sulfur, intermixed with bacteria. On making a transfer into a fresh flask with medium, a sulfur layer was obtained in 24 hours, originating from the thiosulfate. This reaction is exothermic and functions as a source of energy. The energy is used for the reduction of  $\text{NaHCO}_3$  and for the building of the bacterial body. Calcium sulfide, hydrogen sulfide and tetrathionate can replace the thiosulfate. The ammonium salt can be replaced by nitrates. None of the organic substances tested could replace the carbonic acid as a source of carbon. The organism, *Thiobacillus thioparus* Beijerinck, was reported to be a short rod, 3 by  $0.5\mu$ , not forming any spores, very motile and very sensitive, so that on plates the organisms die off in a week.

By adding 2 per cent agar to the above medium *Th. thioparus* can be grown on the plate; transfers are then made from individual colonies into fresh lots of the liquid medium giving a pure culture of the organism. The colonies are of a pin-point form and are distinguished from contaminations by their yellow appearance, due to an abundant separation of sulfur. According to Dügge<sup>54</sup> the bacterium is only 0.3 to  $0.5\mu$  long. Jacobsen<sup>55</sup> has succeeded in bringing about oxidation by this (?) organism of sulfur to sulfuric acid, in the following medium:

$\text{K}_2\text{HPO}_4$ .....	0.5 gram	$\text{CaCO}_3$ or $\text{MgCO}_3$ ....	20.0 grams
$\text{NH}_4\text{Cl}$ .....	0.5 gram	Precipitated sulfur...	10.0 grams
$\text{MgCl}_2$ .....	0.2 gram	Distilled water.....	1000 cc.

An organism similar to the *Thiobacillus thioparus* was found<sup>56</sup> to be active in the oxidation of sulfur in alkali soil, morphologically similar to the form studied by Nathanson and Beijerinck.

This group of sulfur bacteria includes, in addition to the aerobes, also anaerobic bacteria which are able to obtain their oxygen from nitrates. Beijerinck obtained an oxidation of sulfur accompanied by a reduction of the nitrate to atmospheric nitrogen by using the following medium in closed flasks and incubating at 30°C.

$\text{KNO}_3$ .....	0.5 gram	$\text{CaCO}_3$ .....	20.0 grams
$\text{Na}_2\text{CO}_3$ .....	0.2 gram	Sulfur.....	100.0 grams
$\text{K}_2\text{HPO}_4$ .....	0.2 gram	Canal water.....	1000 cc.

<sup>54</sup> Dügge, 1919 (p. 80).

<sup>55</sup> Jacobsen, H. C. *Folia Microb.* 1: 487-496. 1912; 3: 155-162. 1914.

<sup>56</sup> Waksman, S. A. *Jour. Bact.* 7: 609-616. 1922.



The sulfur is oxidized to sulfuric acid which acts upon the  $\text{CaCO}_3$  giving  $\text{CaSO}_4$  and  $\text{CO}_2$ . Beijerinck isolated in pure culture, *Thiobacillus denitrificans*, a very motile, short rod, hardly distinguishable microscopically from *Th. thioparus*, using the following medium:

$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ .....	5.0 grams	Agar.....	20.0 grams
$\text{K}_2\text{HPO}_4$ .....	0.1 gram	Tap water.....	1000 cc.
$\text{NaHCO}_3$ .....	0.2 gram		

On the plate, both organisms lose their ability to grow very rapidly, long before they are dead.

The denitrifying organism was studied in greater detail by Lieske,<sup>57</sup> who used a medium having the following composition:

$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ .....	5.0 grams	$\text{MgCl}_2$ .....	0.1 gram
$\text{KNO}_3$ .....	5.0 grams	$\text{CaCl}_2$ .....	Trace
$\text{NaHCO}_3$ .....	1.0 gram	$\text{FeCl}_3$ .....	Trace
$\text{K}_2\text{HPO}_4$ .....	0.2 gram	Distilled water.....	1000 cc.

This medium is placed in tall glass cylinders and is inoculated with river mud containing  $\text{H}_2\text{S}$ . In a few days, one or more opalescent zones are formed in the liquid, at some distance from the surface, containing the denitrifying bacteria in question. The culture is then transferred into an Erlenmeyer flask filled with the medium and stoppered with a rubber stopper through which a bent glass tube is passed, one end of which is dipped in mercury and the rest filled with medium. The culture is incubated at  $25^\circ$  to  $30^\circ\text{C}$ . and in a few days the active formation of nitrogen gas takes place. By inoculating the above medium, to which 1.5 per cent washed agar has been added, pure cultures are obtained. The organism can also be cultivated in dilute meat extract media to which thiosulfate has been added.

Lieske described *Th. denitrificans* as a small narrow rod,  $1\mu$  long, not producing any spores. It is not injured by sunshine or oxygen, although it thrives better in its absence. It is autotrophic, but is not injured by organic substances. Various carbonates and bicarbonates can be used as sources of carbon, but  $\text{CO}_2$  cannot be used because of the injurious effect of the free sulfuric acid formed. In the presence of nitrates, the following substances can be utilized as sources of energy: hydrogen sulfide, flowers of sulfur, sodium thiosulfate and sodium tetrathionate, which are completely oxidized to sulfate.

<sup>57</sup> Lieske, R. Ber. deut. bot. Gesell. 30: 12-22. 1912.

The energy liberated in the process is sufficient both for the reduction of the nitrate, which is an endothermic phenomenon, and the assimilation of  $\text{CO}_2$  from the carbonate and bicarbonate. The reason why the same organism can oxidize various compounds of sulfur, while other autotrophic soil bacteria, like those concerned in nitrification, can act only upon one definite compound, was explained by Lieske to be due to the step-like oxidation of the sulfur compounds. For every 100 gm. of  $\text{Na}_2\text{S}_2\text{O}_3$  oxidized to sulfate, 1 gm. of carbon was assimilated.

*Th. denitrificans* is of universal occurrence in various soil types, increasing in abundance with an increase in the carbon content of the soil.<sup>58</sup> By adding thiosulfate and bicarbonate to the soil, intensive nitrate decomposition takes place. The organisms occurring in various soils vary in activity, the strain found in composts, forest soils and peat being four times as active as that found in cultivated soils. *Th. denitrificans* offers according to Beijerinck,<sup>59</sup> the natural connecting link between sulfur oxidizing bacteria and denitrifying bacteria.

Trautwein<sup>60</sup> isolated an organism from the soil which was classified with *Th. denitrificans* Beij.; this organism is 1 to 2 by  $0.5\mu$  in size, motile, can reduce nitrate, but can grow also under aerobic conditions; it grows well on organic media and does not precipitate any sulfur from thiosulfate. The organism is facultative autotrophic, since it can obtain its carbon both from  $\text{CO}_2$  (with thiosulfate as a source of energy) and from organic substances (in the absence of thiosulfate). It was grown on the following medium:

$\text{KNO}_3$ .....	1.0 gram	$\text{NaHCO}_3$ .....	1.0 gram
(or $\text{NH}_4\text{Cl}$ ).....	0.1 gram	$\text{MgCl}_2$ .....	0.1 gram
$\text{Na}_2\text{HPO}_4$ .....	0.2 gram	Distilled water.....	1000 cc.
$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ .....	2.0 grams		

To prepare a solid medium, agar is added to the above solution. As organic media, ordinary bouillon or nutrient agar can be employed.

The bacterium was found to be related<sup>61</sup> to the fluorescens group in the Lehmann and Neumann system, especially to the *Bact. denitrificans* (Stutzer and Burri) L and N.

According to Klein and Limberger,<sup>62</sup> the thionic acid bacteria are capable of oxidizing all sources of sulfur found in the soil (elementary

<sup>58</sup> Gehring, A. Centrbl. Bakt. II, **42**: 402-438. 1915.

<sup>59</sup> Beijerinck, 1920 (p. 479).

<sup>60</sup> Trautwein, K. Centrbl. Bakt. II, **53**: 513-548. 1921.

<sup>61</sup> Trautwein, K. Centrbl. Bakt. II, **61**: 1-5. 1924.

<sup>62</sup> Klein, G. and Limberger, A. Biochem. Ztschr. **143**: 473-483. 1923.

sulfur, hydrogen sulfide, other sulfides, sulfites and hydrosulfites) to sulfate and polythionate. The sulfur can also be utilized in the form of organic sulfur (cystin, albumin, nuclein, meat extract) and is oxidized, through the sulfur stage, to sulfate. The organism reduces  $\text{KNO}_3$  to nitrite and to ammonia, in the oxidation of sulfur. The claim, however, that this organism can also oxidize  $\text{NH}_4\text{Cl}$  to nitrite would tend to indicate that this investigation needs further confirmation.

MINUTE, COLORLESS, AEROBIC, NON-SPORE FORMING AND NON-THREAD FORMING BACTERIA, ACTING PRIMARILY ON ELEMENTARY SULFUR AND OXIDIZING IT RAPIDLY TO SULFURIC ACID (No. 24, Pl. III). When sulfur is mixed with soil, it is oxidized slowly at first and then as the soil becomes acid it oxidizes rapidly. If powdered rock phosphate is added to the mixture of soil and sulfur, the rock is transformed into soluble phosphates by the acid formed from the sulfur. This process has been utilized<sup>63</sup> in composting sulfur, rock phosphate and soil in various proportions. A direct correlation was found between the acid formed, as shown by the increase in the hydrogen-ion concentration, and the amount of phosphates going into solution. When a fresh compost is inoculated with some material from an old compost, the reaction goes on more rapidly, indicating the biological nature of the process.

By inoculating a medium free from any organic compounds and carbonates and containing sulfur as the only source of energy, in addition to minerals and tri-calcium phosphate as a neutralizing agent, the growth of a bacterium, capable of oxidizing sulfur to sulfuric acid, was obtained.<sup>64</sup> The acid produced interacted with the tricalcium phosphate and transformed it into  $\text{CaSO}_4$  and monocalcium phosphate and finally into phosphoric acid. The bacterium was, however, accompanied by other organisms, chiefly mold spores, which persisted in the medium on repeated transfer. Repeated attempts to grow the bacterium on agar plates failed. After all the calcium of the phosphate had been transformed into calcium sulfate, the medium became very acid, as low as pH 0.58.

In order to make use of the fact that the organism can withstand a high acid concentration, the media were prepared with an initial reaction of pH 2.0. This allowed only the development of the sulfur-oxidizing organism. The high initial acidity accompanied by the use of high dilutions of the culture in making the transfers (1:100,000), finally

<sup>63</sup> Lipman, J. G., McLean, H. C. and Lint, H. C. *Soil Sci.* **2**: 499-538. 1916.  
McLean, H. C. *Soil Sci.* **5**: 251-290. 1918.

<sup>64</sup> Lipman, J. G., Waksman, S. A. and Joffe, J. S. *Soil Sci.* **12**: 475-489. 1921.

resulted in obtaining the culture pure. This was demonstrated by the fact that no growth took place when inoculated on bouillon and other media, favorable for the development of bacteria and fungi, even after 10 to 14 days incubation. Microscopic examinations also demonstrated the purity of the culture. This organism was described as *Thiobacillus thiooxidans*.<sup>65</sup> It is a small, non-motile organism, 0.75 to 1.0 by 0.5 to 0.75 $\mu$ , producing cloudiness throughout the medium but without the formation of any pellicle. The medium best adapted for the growth of this organism has the following composition:

(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .....	0.2 gram	CaCl <sub>2</sub> .....	0.25 gram
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.5 gram	Elementary, pow-	
KH <sub>2</sub> PO <sub>4</sub> .....	3.0 grams	dered sulfur.....	10 grams
		Distilled water.....	1000 cc.

The sulfur is weighed out separately and the medium is sterilized for thirty minutes, in flowing steam, on three consecutive days. When this medium is inoculated with a fresh vigorous culture (seven to fourteen days old) of the *Th. thiooxidans*, growth will be manifested by a uniform turbidity, without any pellicle formation, within four to five days, at 25 to 30°C., the culture becoming very turbid in seven to eight days; the sulfur which has been floating on the surface begins to drop down. The same phenomenon is observed when the medium is inoculated with a little soil containing the bacterium, only the length of time required for development is sometimes a little longer, depending upon the abundance of the organism in the soil, condition of soil, etc. By using the dilution method, even the approximate number of the organisms in the soil can be estimated. The culture is practically pure, due to the fact that very few other organisms would develop under these conditions.

The bacterium is strictly aerobic and is benefited both by aeration and greater surface exposure. When a particle of sulfur from the flask is examined, it is found to be surrounded by the bacteria. At the same time there is an intense increase in acidity of the medium.

In the presence of calcium phosphate or carbonate, the sulfuric acid, as soon as formed, interacts with the calcium salt giving crystals of CaSO<sub>4</sub>·2H<sub>2</sub>O, which are seen in the culture hanging down from the particles of sulfur floating on the surface, till finally the bottom of the flask is covered with gypsum crystals. The organism forms no spores and is destroyed at 55° to 60°C. in several minutes. The limiting alkaline reaction is about pH 6.0, which is distinctly acid, while at the other extreme it will grow at pH 1.0. The optimum lies at pH 2.0 to 4.0. It is possible, however, to accustom the organism to a neutral and even an alkaline reaction, especially when transferred from one soil to another before the reaction becomes too acid.

<sup>65</sup> Waksman, S. A. and Joffe, J. S. Jour. Bact. 7: 239-256. 1922.

The organism derives its carbon from the  $\text{CO}_2$  of the atmosphere; carbonates and bicarbonates affect it injuriously in so far as they tend to make the reaction alkaline. The presence of organic substances is not injurious. As a matter of fact, sugars, like lactose and galactose, ethyl alcohol, and glycerol may even slightly stimulate growth but without affecting sulfur oxidation and carbon assimilation. For establishing the purity of the culture, the organism can be grown on a solid medium having the following composition:<sup>66</sup>

$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ .....	5.0 grams	$\text{CaCl}_2$ .....	0.25 gram
$\text{KH}_2\text{PO}_4$ .....	3.0 grams	Agar.....	20.0 grams
$\text{NH}_4\text{Cl}$ .....	0.1 gram	Distilled water.....	1000 cc.
$\text{MgCl}_2$ .....	0.1 gram		

The medium is prepared as usual and sterilized at 15 pounds pressure for fifteen minutes. Plates and slants are inoculated from a vigorous liquid culture and incubated at  $25^\circ$  to  $30^\circ\text{C}$ . Growth appears in five to six days in the form of minute straw yellow to cream-colored colonies. Under the microscope, each colony is found to be surrounded with crystals of gypsum due to the action of the sulfuric acid, formed from the oxidation of the thiosulfate, upon the  $\text{CaCl}_2$ . This phenomenon is particularly prominent in media containing tri-calcium phosphate in place of the chloride; a clear zone is formed around each colony, due to the disappearance of the insoluble calcium salt.

When, instead of an ordinary neutral or acid soil, an alkaline soil is used for composting, the sulfur is also oxidized to sulfuric acid with the result that the alkalinity of the soil is decreased. When enough sulfur is added, black alkali soil having a reaction of pH 9.8 can be made neutral and even acid. At first it was thought that *Thiobacillus thiooxidans* is responsible for this oxidation, particularly since the same acid composts were employed. It is possible, however, that the organism concerned in the oxidation of elementary sulfur in alkali soil is of an entirely different nature, approaching more the *Th. thioparus* of Beijerinck, in its cultural and some physiological characters, rather than the *Th. thiooxidans*. The latter, however, is also found in alkaline composts, and it is possible that both organisms take an active part in the oxidation of the sulfur under alkaline conditions. The *Th. thioparus* group has its optimum on the alkaline side (pH 7.0 to 9.0), as shown by Trautwein, while the *Th. thiooxidans* has its optimum on the acid side. Both organisms may, therefore, act upon the sulfur under alkaline conditions. *Th. thiooxidans* has been found in Europe, in very acid sandy

<sup>66</sup> Waksman, S. A. Jour. Bact. 7: 605-608. 1922.

soils of Denmark originating from a dry sea bottom, in acid soils in Germany, in India, Russia and elsewhere.<sup>67</sup>

A number of other species of *Thiobacillus* were described; these were isolated from sea water, from mud of sulfur springs and other sources. Most of the descriptions of these organisms are not sufficient for purposes of identification. Their phylogenetic relationships, as well as differences in chemical action upon different sulfur compounds still remain to be investigated.

*Oxidation of selenium and its compounds.* Brenner<sup>68</sup> isolated from the soil an organism (*Micrococcus selenicus*, less than  $0.5\mu$  in size), which is capable of oxidizing selenides and of using the energy obtained for its activities. Sodium selenite, sodium thiosulfate or sodium selenate, as well as litmus, methylene blue or indigo carmin, can be used as hydrogen acceptors (or sources of oxygen) but not nitrates, sulfates, sulfites or tellurites. In addition to selenide, the organism can also use various alcohols (ethyl-, iso-butyl) as well as asparagine and glucose as sources of energy, but not proteins. The carbon is obtained, however, only from organic substances (ethyl alcohol). The possible oxidation of elementary selenium, in the absence of organic matter, with an increase of acidity of the medium has also been suggested.<sup>69</sup> The *Th. thioparus* and *Th. thiooxidans* were found to be inactive in both cases. The relation between the oxidation of selenium and of selenide and autotrophic processes is unknown.

*Bacteria oxidizing iron compounds.* The strictly iron bacteria, or those organisms that are capable of oxidizing ferrous to ferric iron, whereby the energy obtained is used for the chemosynthetic assimilation of carbon, should be distinguished from those bacteria that can absorb or accumulate iron, when living in media containing iron, or can bring about the precipitation of the iron due to the changes in the reaction of the medium. Unlike the latter process the precipitation of iron by true iron bacteria is a direct result of utilization of energy from the oxidation of iron.<sup>70</sup>

As early as 1836, Ehrenberg<sup>71</sup> found that microorganisms play an

<sup>67</sup> Jensen, H. L. Centrbl. Bakt. II, 72: 242-246. 1927; Brown, H. D. Jour. Amer. Soc. Agron. 15: 350-382. 1923; Aiyar, C. V. R. and Norris, R. V. Jour. Indian Inst. Sci. 12A: 274-294. 1929; Drewes, 1928 (p. 40). Lange-Pozdeieva, I. P. Arch. Biol. Nauk (Russian), 30: 189-201. 1930.

<sup>68</sup> Brenner, W. Jahrb. wiss. Bot. 57: 95-127. 1916.

<sup>69</sup> Lipman, J. G. and Waksman, S. A. Science, N. S. 57: 58. 1923.

<sup>70</sup> Winogradsky, 1922 (p. 60).

<sup>71</sup> Ehrenberg, G. C. Poggendorff's Annalen 38: 213-227. 1836.

important part in the formation of ochraceous deposits of bog iron ore. The iron precipitating organisms are present universally in nature, wherever iron-bearing waters occur. They belong chiefly to the thread-forming bacteria, although a number of them have also been found to belong to the Eubacteria. Here again we find a similarity between the iron and sulfur bacteria, a large number of forms belonging to distinctly different morphological groups. The majority of these forms belong to the higher bacteria, according to the following classification<sup>74</sup>:

I. Thread-forming bacteria consisting of sheaths with included cells generally plainly visible. Reproduction by internally produced conidia or by the separation of motile and non-motile cells (Trichobacteria): *Crenothrix*, *Leptothrix*, *L. ochracea*, *L. trichogenes*.

II. True bacteria: *Gallionella* (*Spirophyllum*), *Siderocapsa*, *Sideromonas*.

Only a few of the numerous forms described are true iron bacteria.

It was assumed that the higher bacteria present a variety of forms, such as single threads composed of cylindrical cells placed end to end and generally inclosed in sheaths; ribbon forms, twisted spirally; cylindrical threads showing false branching, or coiled threads and ribbon forms produced by the bending of the filaments in the middle and the twisting of the ends around each other like a rope.<sup>72</sup> *Gallionella*<sup>73</sup> was considered to be the most abundant of the iron bacteria. The flat ribbon-like or tape-like threads are twisted in the form of spirals. These spiral bands may occur as single filaments or may be coiled together. Cholodny<sup>74</sup> demonstrated, however, that these spiral filaments consist of ferric oxide excreted by small bacteria, a single individual organism being found at the end of each filament. The threads are thus formed as a result of the oxidizing capacity of the organism followed by excretion of the product of oxidation. On treatment with hydrochloric acid, the filaments dissolve leaving the living bacteria at the end. These bacteria are only  $1.2 \times 0.5\mu$  in size and consist of two coccus-like cells,  $0.6$  by  $0.5\mu$ .

Naumann<sup>75</sup> considered as iron organisms all those that take an active

<sup>72</sup> Harder, E. C. Prof. paper 113, U. S. Geological Survey, Dept. of Interior, 1919.

<sup>73</sup> Ellis, D. Proc. Roy. Soc. Edinburgh, 27: 21-34. 1907.

<sup>74</sup> Cholodny, N. Die Eisenbakterien. G. Fischer, Jena. 1926; Jour de microb. (Russian) 9: 149-158, 1929; Planta, Arch. wiss. Bot., 8: 252-268. 1929.

<sup>75</sup> Naumann, E. Centrbl. Bakt. II, 78: 512-515. 1919; Ber. deut. chem. Gesell. 46: 135. 1928; Intern. Rev. Hydrob. Hydrogr. 24: 81-96. 1930.

part in the cycle of iron in nature. These organisms were classified as follows:

I. Those that precipitate iron or *siderogones*: (1) *Siderophores* or those that accumulate iron; these include the strictly autotrophic organisms. (2) Non-iron accumulating forms.

II. Those that bring about the dissolution of iron or *siderophages*.

Some of the iron bacteria are also capable of oxidizing manganese salts and precipitate manganese hydrates in their cells;<sup>76</sup> Winogradsky suggested that we may be dealing here with organisms less specialized than the other autotrophic bacteria, some being iron-bacteria in the proper sense (*Gallionella*), some iron-manganese bacteria (*Crenothrix*, *Leptothrix*), and some may possibly be obligate manganese-bacteria.

Winogradsky<sup>77</sup> found in 1888 that *Leptothrix* will live and grow only in solutions in which iron is present in the ferrous form; where the living cells are present, a brown coloration of the sheath takes place due to the oxidation of the iron salt. The oxidation of ferrous compounds ( $\text{FeCO}_3$ ) to ferric hydroxide is necessary for the life and growth of the organisms, this process furnishing the necessary energy to the cell for the assimilation of carbon. According to Molisch,<sup>78</sup> however, *Leptothrix* will grow well in iron-free media, particularly in peptone solutions, forming perfectly colorless sheaths. If iron or manganese compounds are present in the solutions, they are oxidized and taken up by the sheaths; even dead cells (killed by boiling) are able to take up ferric hydroxide in their sheaths. He, therefore, concluded that the process is merely physico-chemical and the change of ferrous to ferric compounds is due to simple chemical oxidation and is not connected with the life processes of the cell. Similar observations were made by Ellis and others,<sup>79</sup> who claimed that these bacteria living in iron waters have the power of attracting ferric hydroxide, which is found in quantity in such waters, the deposition of iron being merely a purely mechanical process. This was entirely due to the lack of proper differentiation between the physico-chemical absorption and chemico-biological oxidation of iron, on the one hand, and between the obligate and facultative autotrophy, on the other hand. This led to the general terminology of bacteria which accumulate iron

<sup>76</sup> Schorler, B. *Centrbl. Bakt.* II, 12: 681-695. 1904; 15: 564-568. 1906.

<sup>77</sup> Winogradsky, S. *Bot. Ztg.* 46: 262-270. 1888.

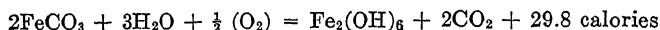
<sup>78</sup> Molisch, H. *Die Eisenbakterien*. Jena. 1910.

<sup>79</sup> Ellis, D. *Centrbl. Bakt.* II, 19: 507-518. 1907; 26: 321-329. 1910; 31: 499-504. 1911; *Science Progr.* 10: 374-392. 1916; *Iron bacteria*. Methuen & Co., London. 1919; Rullman, W. *Centrbl. Bakt.* II, 33: 277-289. 1912; Zikes, H. *Centrbl. Bakt.* II, 43: 520-552. 1915.



as "iron bacteria,"<sup>80</sup> and frequently also to absurdities, as in the case of isolation of a bacterium which can accumulate both iron and calcium (incrustations), where it has been suggested that iron can be replaced by calcium, although evidently no oxidation of calcium is possible.<sup>81</sup>

Lieske<sup>82</sup> found that *Spirophyllum* (*Gallionella*) brings about the oxidation of  $\text{FeCO}_3$  according to the following equation:



Lieske used a medium having the following composition:

$(\text{NH}_4)_2\text{SO}_4$ .....	1.5 grams	$\text{K}_2\text{HPO}_4$ .....	0.05 gram
KCl.....	0.05 gram	$\text{Ca}(\text{NO}_3)_2$ .....	0.01 gram
$\text{MgSO}_4$ .....	0.05 gram	Distilled water.....	1000 cc.

The medium was placed in Erlenmeyer flasks (100 cc.) to a height of 2 cm., sterilized and allowed to stand two days. Coarse iron filings, which had been dry sterilized for one hour at  $160^\circ\text{C}$ . were then added, 0.05 gram to each flask. The flasks were inoculated with a small amount of culture of iron bacteria, placed under a bell-jar in a cool place, and  $\text{CO}_2$  added up to 1 per cent. This resulted in the formation of about 0.01 per cent of  $\text{FeCO}_3$ , which remained constant as long as there was metallic iron present.

*Spirophyllum* developed in four days in the cultures to which iron was added. Pure cultures were obtained by repeated transfer to sterile flasks. Similar results were obtained in 1888 by Winogradsky for *Leptothrix ochracea*, although he did not work with pure cultures. Organic matter in concentrations of over 0.01 per cent (peptone, asparagine, sugar) produced an injurious effect upon the growth of *Spirophyllum*. This organism can oxidize no other iron salt, except the bicarbonate, and also not  $\text{MnCO}_3$ , showing it to be strictly autotrophic and highly specialized. *Leptothrix ochracea* was found by Lieske to be able to utilize manganese carbonate as well as iron carbonate as a source of energy and to be facultative autotrophic, capable of existing also in organic media.

The two media used by Lieske for pure culture study have the following composition:

		II	
Distilled water.....	1000 cc.	Manganese car-	
Agar.....	10.0 grams	bonate saturated	
Manganese acetate...	0.1 gram	solution.....	1:10
		$\text{NaHCO}_3$ .....	0.001 per cent
		$(\text{NH}_4)_2\text{SO}_4$ .....	0.001 per cent
		$\text{K}_2\text{HPO}_4$ and	
		$\text{MgSO}_4$ .....	Traces

<sup>80</sup> Löhnis, 1910, p. 704 (p. xiv).

<sup>81</sup> Brusoff, A. Centrbl. Bakt. II, 45: 547-554. 1916; also 48: 193-210. 1918.

<sup>82</sup> Lieske, R. Jahrb. wiss. Bot. 49: 91-127. 1911; Centrbl. Bakt. II, 49: 413-425. 1919.

One may well agree with Harder<sup>72</sup> that certain iron-depositing organisms, such as *Gallionella*, require ferrous bicarbonate in solution and cannot live without it (obligate autotrophic); others, like *Leptothrix*, can live without any iron compounds, but, if they are present, can use either ferrous bicarbonate (or manganese bicarbonate) or soluble organic compounds (facultative autotrophic); still others, such as the various lower bacteria, will use the organic radical of certain soluble organic iron salts when present but cannot utilize any inorganic iron salts; in other words, the accumulation or incrustation of iron is purely mechanical and these bacteria should not be considered as iron bacteria at all but merely as heterotrophic organisms. One may include among the latter the spore-forming bacterium<sup>83</sup> which precipitates ferrie hydroxide from solutions of iron salts, then reduces the hydroxide anaerobically to bog iron.

*Bacteria obtaining their energy from the oxidation of methane.* Methane may be produced in appreciable amounts in volcanic eruptions, around oil mines and as a result of different chemical processes. It is also produced in the anaerobic decomposition of cellulose, of other carbohydrates, organic acids and alcohols, as well as proteins.<sup>84</sup> Swamps, manure heaps and low-lying meadows also contribute large amounts of methane to the atmosphere.

Although the chemical oxidation of methane has been demonstrated in various instances, it is primarily a phenomenon accomplished by microorganisms. According to Harrison and Aiyer,<sup>85</sup> the soil film contains bacteria capable of oxidizing methane and hydrogen and assimilating methane and CO<sub>2</sub>, increasing the oxygen output. *B. methanicus* was isolated from the soil by Söhngen.<sup>86</sup> It was found to be a short, motile rod, 2 to 3 by 1.5 to 2  $\mu$  in size, and could transform methane partly into organic compounds and partly into CO<sub>2</sub>. In older cultures the organism became nearly spherical.

The medium used by Söhngen consisted of:

Distilled water.....	1000 cc.
K <sub>2</sub> HPO <sub>4</sub> .....	0.05 gram

<sup>83</sup> Mumford, E. M. Jour. Chem. Soc. **103**: 645-650. 1913.

<sup>84</sup> Omeliansky, V. L. Arch. Sci. Biol. St. Petersburg, **12**: No. 2. 1906; Ann. Inst. Past. **30**: 56-61. 1916.

<sup>85</sup> Harrison, W. H. and Aiyer, P. A. S. Mem. Dept. Agr. India, Chem. Ser. **4**: 1-18. 1914.

<sup>86</sup> Söhngen, N. L. Centrbl. Bakt. II, **15**: 513-517. 1906; Hasemann. Biochem. Ztschr. **184**: 147-171. 1927.

MgNH <sub>4</sub> PO <sub>4</sub> ·6H <sub>2</sub> O.....	0.1 gram
CaSO <sub>4</sub> .....	0.01 gram

Inoculation was made with soil. The atmosphere consisted of one part CH<sub>4</sub> and two parts air.<sup>87</sup>

Certain other common bacteria are capable of oxidizing methane, as in the case of *Bact. pyocyaneum* and *Bact. fluorescens liquefaciens*. The methane-oxidizing bacteria were found to be distributed in greater numbers in the deeper layers of soil than close to the surface.<sup>88</sup> Münz<sup>89</sup> isolated a methane oxidizing organism, not identical with that of Söhngen, which he also called *Methanomonas methanica*. It grew at 18° to 40° with an optimum at 34°C. This organism measured 0.9 to 2.2 by 0.3 to 0.4 $\mu$  in size, was elliptical to cylindrical, non-motile. High methane and low oxygen content of atmosphere were best for its growth, although the organism was aerobic. Hydrogen and carbon monoxide could not replace methane, although alcohols, carbohydrates and salts of organic acids could be used. Nitrogen was utilized both in inorganic and organic forms. The organism may be considered as facultative autotrophic, although the autotrophy of this organism is still questionable.

*B. hexacarbovorum* was found to be able to utilize methane, toluol, xylol and illuminating gas as the only sources of carbon. Various other hydrocarbons can also be utilized as sources of energy by bacteria.<sup>90</sup> In this connection mention should be made of the work of Söhngen on the Mycobacteria (*Mycob. lacticola*, *Mycob. phlei*), which were found capable of deriving their energy from the oxidation of benzol, paraffin, petroleum, and assimilating the CO<sub>2</sub> of the atmosphere. In regard to the oxidation of pure carbon by bacteria, certain investigations<sup>91</sup> point to positive and others<sup>92</sup> to negative results.

*Bacteria oxidizing carbon monoxide.* Carbon monoxide is produced, in large amounts, in the incomplete combustion of carbon compounds

<sup>87</sup> Kaserer, H. Centrbl. Bakt. II, 15: 573-576. 1906.

<sup>88</sup> Söhngen, N. L. Proefschrift. Delft. 1906 (Bot. Centrbl. 105: 371-372. 1907); Giglioli and Masoni. Staz. sper. Agr. Ital. 42: 589. 1909 (Chem. Centrbl. I, 1910, 294); Aiyer, P. A. S. Mem. Dept. Agr. India, Chem. Ser. 5: 177-180. 1920.

<sup>89</sup> Münz, E. Zur Physiologie der Methanbakterien. Diss. Halle, 1915.

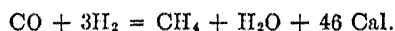
<sup>90</sup> Störmer, 1907 (p. 47); Tausz, J. and Peter, M. Centrbl. Bakt. II, 49: 497-554. 1920.

<sup>91</sup> Galle, Centrbl. Bakt. II, 28: 461-473. 1910; Lieske, R. and Hoffmann, E. Brennstoffchemie. 9: 174-178. 1928.

<sup>92</sup> Schroeder, H. Centrbl. Bakt. II, 41: 460-469. 1914.

and, in small amounts, in the decomposition of manure.<sup>93</sup> It can be oxidized readily in contact with soil organic matter.<sup>94</sup> When the soil is sterilized by means of heat or disinfectants, its ability to oxidize carbon monoxide is practically stopped. The organism responsible for this process has been identified with the *Bac. oligocarbophilus* Beij.,<sup>95</sup> a non-motile, non-spore forming rod,  $0.5 \times 0.5-4.0\mu$  in size, and producing a snow-white, dry pellicle on the surface of liquid media. The organism can live autotrophically with CO or illuminating gas as the only source of energy, but also heterotrophically upon organic media. This bacterium has later<sup>96</sup> been identified as an actinomycetes.

According to Lieske,<sup>97</sup> the process of oxidation of carbon monoxide by bacteria is exothermic and can serve as a source of energy:



Growth of the bacteria takes place in inorganic solutions, and is favored by small amounts of inorganic or organic colloids. A long, Gram-positive, non-spore forming and non-motile bacterium, related to Söhngen's organism, was isolated.

Carbon monoxide seems to be oxidized also under anaerobic conditions, whereby sulfates are used as sources of oxygen; the nature of the organism responsible for this process is still unknown. The oxidation of illuminating gas is brought about not by a single organism but by a mixture of at least four organisms capable of oxidizing carbon monoxide, methane and hydrogen.<sup>97a</sup>

*Bacteria oxidizing hydrogen.* Hydrogen can be oxidized both aerobically and anaerobically. De Saussure<sup>98</sup> demonstrated in 1838 that moist soil will transform hydrogen readily into water, while soil heated or treated with antiseptic substances is unable to do so. A number of bacteria were isolated<sup>99</sup> from soils which were able to oxidize hydrogen

<sup>93</sup> Löhnis, 1910 (p. xiv).

<sup>94</sup> Wehmer, C. Ber. deut. Chem. Gesell. 59: 887. 1926.

<sup>95</sup> Beijerinck, M. W. and Van Delden, A. Centrbl. Bakt. II, 10: 33-47. 1903.

<sup>96</sup> Lantzsch, K. Centrbl. Bakt. II, 57: 309-319. 1922.

<sup>97</sup> Lieske, R. and Hoffmann, E. Brennstoffchem. 11: 208-212, 1930; Biochem. Ztschr. 236: 247. 1931; Söhngen, N. L. Rec. Trav. Chim. Pays Bas, 29: 238. 1910.

<sup>97a</sup> Hasemann, W. Biochem. Ztschr. 184: 147-171. 1927.

<sup>98</sup> deSaussure, Th. Mem. Soc. phys. Hist. Nat. Genève, 8: 163-190. 1839; Immendorff, H. Landw. Jahrb. 21: 281-339. 1892.

<sup>99</sup> Kaserer, H. Centrbl. Bakt. II, 16: 681-696, 769-775. 1906; Niklewski, B. Centrbl. Bakt. II, 20: 469-473. 1908; Jahrb. Wiss. Bot. 48: 113-142. 1910. Nabokich, A. J. and Lebedeff, A. F. Centrbl. Bakt. II, 17: 350-355. 1906; Biochem. Ztschr. 7: 1-10. 1908.

autotrophically with the formation of water. The organism isolated from the soil by Kaserer, namely *Bac. pantotrophus*, was an aerobic, short, motile rod, 1.2 to 1.5 by 0.4 to 0.5 $\mu$  in size, occurring singly or in chains, encapsulated. It was motile by means of a single polar flagellum. The gelatin colonies were yellow, smooth, rarely greenish; the gelatin was not liquefied. Yellow to greenish growth on agar.

Kaserer suggested that both the methane and hydrogen oxidation phenomena take place in the soil, due to the fact that these substances, which are produced in the subsoil by anaerobic processes, are thus oxidized.

Kaserer's medium consisted of:

K <sub>2</sub> HPO <sub>4</sub> .....	0.5 gram	NaHCO <sub>3</sub> .....	0.5 gram
MgSO <sub>4</sub> .....	0.2 gram	FeCl <sub>3</sub> .....	Trace
NH <sub>4</sub> Cl.....	1.0 gram	Water.....	1000 cc.

The organism growing on this medium developed poorly under autotrophic conditions, the oxidation of hydrogen becoming prominent in the presence of small amounts of soluble organic matter.<sup>100</sup> A non-motile bacterium, 1.4 by 0.5 $\mu$  in size, was isolated by Harrison and Aiyer, different from the *Bac. pantotrophus* of Kaserer. The greatest amount of hydrogen was oxidized in the presence of 0.01 to 0.03 per cent peptone, nutrose or sodium asparaginate. In association with certain bacteria, the organism was much more active.

Niklewski used a medium containing:

NH <sub>4</sub> Cl.....	1.0 gram	NaCl.....	0.2 gram
KH <sub>2</sub> PO <sub>4</sub> .....	1.0 gram	FeCl <sub>3</sub> .....	0.0001 gram
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.2 gram	Agar.....	15.0 grams
NaHCO <sub>3</sub> .....	1.0 gram	Water.....	1000 cc.

The cultures were placed in a bell-jar, through which purified hydrogen was passed, at 38° to 35°C. The cultures developed in 3 to 4 days. Two organisms were isolated:

*Hydrogenomonas vitrea* formed a pellicle on the surface of the liquid medium. Small yellow subsurface colonies were formed on the agar. On the surface the colonies were transparent, folded. The cells were 2 $\mu$  long. Obligate autotrophic. No motility observed.

*H. flava* formed shining yellow colonies on the surface of the agar, not spreading as rapidly as the *H. vitrea*, surface smooth, edge entire; microscopically, the cells were found to be somewhat smaller (1.5 $\mu$  long). No pellicle formation on liquid media. Obligate autotrophic. No motility observed.

<sup>100</sup> Harrison, W. H. and Aiyer, P. A. S. Mem. Dept. Agr. India, Chem. Ser. 135-148. 1916.

By further study, Niklewski<sup>101</sup> isolated a bacterium (*H. agilis*) which can oxidize hydrogen by using the oxygen obtained from the reduction of nitrates and sulfates and in some cases of citrate, tartrate and oxalate. The presence of nitrate enables the organism to oxidize hydrogen anaerobically.<sup>102</sup> The bacterium can exist aerobically using free oxygen for the autotrophic oxidation of hydrogen. It can also develop heterotrophically similar to the other organisms.

For the isolation of pure cultures of hydrogen oxidizing bacteria, Lebedeff<sup>103</sup> used a medium consisting of 1000 parts of water, 2.0 KNO<sub>3</sub>, 0.5 NaH<sub>2</sub>PO<sub>4</sub>, 0.2 MgSO<sub>4</sub>, traces of FeCl<sub>3</sub> and an atmosphere of hydrogen containing 5 to 15 per cent CO<sub>2</sub>. One hundred cubic centimeter portions of medium were placed in side-arm flasks, inoculated with soil; after evacuating and introducing the gas, the flasks were sealed. After 5 to 6 days of growth the organism appeared in the form of a surface pellicle. After 3 to 4 transfers, the organism was isolated on a silica gel plate, kept in an atmosphere of hydrogen containing 5 to 10 per cent CO<sub>2</sub>. After 8 to 10 days, milky-white regular colonies appeared on the plate. These consisted of rod-shaped bacteria, 1.2 to 1.5 $\mu$  long, motile by means of a single flagellum. The organism was heterotrophic, forming on gelatin, milky-white colonies which changed later to brownish, and was named *Bac. hydrogenes*. Gelatin was liquefied, various sugars, organic acids and protein derivatives were utilized as sources of carbon. Optimum temperature was found to be 26°C.

More detailed studies<sup>104</sup> have shown that a number of different species of hydrogen bacteria are present in the soil. They live autotrophically with hydrogen as a source of energy and heterotrophically in the absence of hydrogen, thus being facultative autotrophic. The different species differ in their sensitiveness to oxygen pressure or in the ability to use combined oxygen for the oxidation of hydrogen. A newly found species *Bacillus pycnoticus* was studied in detail. It is a rod-shaped organism, 1.5 to 4 by 1.0 $\mu$ , every preparation containing non-motile and motile cells, with peritrichic flagellation. In addition to the rods, true cocci as well as giant cells and thick-walled, egg-shaped cells, ten times as large as the normal bacterial cell, were found in the culture. The purity of the culture was established by single-cell isolation, using Burri's India ink method. The giant cells are involution forms, produced under special environmental conditions. The spores swell up in length and width,

<sup>101</sup> Niklewski, B. Kosmos, Lemberg. 1923. (Centrbl. Bakt. II, 40: 430-433. 1914.)

<sup>102</sup> Beijerinck, M. W. and Minkman, D. C. J. Centrbl. Bakt. II, 25: 30-63. 1910; Grzymirska, H. Acta Soc. Bot. Pol. 5: No. 6. 1928.

<sup>103</sup> Lebedeff, A. F. Investigations of the chemosynthesis of *Bacillus hydrogenes* (Russian). Odessa. 1910.

<sup>104</sup> Grohmann, G. Centrbl. Bakt. II, 61: 256-271. 1924; Ruhland, W. Jahrb. Wiss. Bot. 63: 321-389. 1924.

TABLE 15  
Summary of physiological properties of autotrophic bacteria

	NAME OF ORGANISM	SOURCE OF ENERGY	INFLUENCE OF ORGANIC SUBSTANCES	CARBON SOURCE	NITROGEN SOURCE	TEMPERATURE LIMIT	APPROXIMATE OPTIMUM REACTION	SIZE	AUTHORS
Bacteria oxidizing simple nitrogen compounds	<i>Nitrosomonas Nitrosococcus</i>	NH <sub>4</sub> salts	Injurious, not utilized	CO <sub>2</sub>	NH <sub>4</sub> salts	5-55 Opt. 34	pH 7.7-7.9	μ 1.2-1.8 x 0.9-1.0 1.4-1.7	Winogradsky, Gaarder and Hagen
	<i>Nitrobacter</i>	Nitrites	Not injurious (small amounts), not utilized	CO <sub>2</sub>	Nitrite	5-55 Opt. 34	6.8-7.3	1 x 0.3-0.4	Winogradsky, Gaarder and Hagen
Bacteria oxidizing sulfur and its inorganic compounds	<i>Bagiataea and Thiobacillus</i>	H <sub>2</sub> S	Without influence on metabolism	CO <sub>2</sub>	NH <sub>4</sub> salts	0-45 Opt. 30	8.0 (?)	40-200 x 0.8-5.0	Winogradsky and Keil
	<i>Thiobacillus thioparvus</i>	NasS <sub>2</sub> O <sub>3</sub> ·5 H <sub>2</sub> O	Without influence on metabolism	CO <sub>2</sub>	Nitrate or NH <sub>4</sub> Cl		8.0 (?)	3 x 0.5	Nathanson and Beijerinck
	<i>Thiobacillus denitrificans</i>	H <sub>2</sub> S, sulfur, NasS <sub>2</sub> O <sub>3</sub> , tetrathionate	Can be utilized	CO <sub>2</sub>	Nitrate	Opt. 30	7.0-9.0	1-2 x 0.5	Beijerinck, Lieske, Trautwein
	<i>Thiobacillus thiooxidans</i>	Sulfur, thiosulfate, sulfides	Without influence	CO <sub>2</sub>	NH <sub>4</sub> salts	0-40 Opt. 30	0.5-6.0 Opt. 3.0	0.75-1 x 0.5	Waksman and Joffe
Iron bacteria	<i>Leptothrix ochracea</i> <i>Gallionella ferruginea</i>	FeH <sub>2</sub> (CO <sub>3</sub> ) <sub>2</sub> FeH <sub>2</sub> (CO <sub>3</sub> ) <sub>2</sub>	Can be utilized Injurious about 0.2%	CO <sub>2</sub> can be utilized CO <sub>2</sub>	Nitrate Nitrate	5-40 Opt. 24 0-22	?	0.6 x 0.5	Winogradsky Lieske
Hydrogen bacteria	<i>Bacillus pycnoliticus</i>	H <sub>2</sub>	Can be utilized	CO <sub>2</sub> and organic carbon	NH <sub>4</sub> Cl, nitrates	Opt. 30	6.8-8.7	1.5-4 x 1.0	Rubland and Grohmann
Methane bacteria	<i>Methanomonas methanica</i>	CH <sub>4</sub>	Can be utilized	CH <sub>4</sub>	Organic N, ammonium salts and nitrates	18-40	?	0.9-2.2 x 0.3-0.4	Münz

before they can germinate, and may account for the egg-shaped figures; they also break up into true cocci.

The bacterium is grown in an inorganic solution containing sufficient iron, the latter being added to the sterilized medium; hydrogen is furnished in the atmosphere over the culture. The optimum reaction is at pH 6.8 to 8.7, the limits being pH 5.2 to 9.2. Growth takes place on the surface, at the boundary between the gas and the liquid; in some cases, the liquid becomes turbid. Partial pressure of the gases ( $H_2$  and  $CO_2$ ) has an inappreciable influence upon growth.

A summary of the physiological characters of the various autotrophic bacteria is given in Table 15.



## CHAPTER V

### BACTERIA FIXING ATMOSPHERIC NITROGEN

*Nitrogen fixation in nature.* All higher plants, all animals and the great majority of microorganisms depend for their nutrition on combined nitrogen, whether organic or inorganic in nature, and can make no use whatsoever of the great store of gaseous nitrogen in the atmosphere. Large quantities of nitrogen, therefore, are removed every year from the soil by the growing crops. In addition to that, several groups of soil microorganisms are even capable of reducing nitrates and liberate atmospheric nitrogen. The quantities of manure returned to the soil are far from sufficient to replace the losses from the soil; the attempt to replace this loss by artificial fertilizers may be sufficient to supply the need of the growing plant but not to replenish the losses from the soil. This is accomplished through the agency of nitrogen-fixing bacteria, working alone or in symbiosis with higher plants. A small amount of combined nitrogen is formed by chemical agencies, such as electrical discharges, and is brought down with the yearly rainfalls, but this hardly amounts to more than a few pounds of nitrogen per acre per year, while ordinary forest trees may remove in the wood and leaves over 50 pounds of nitrogen per acre per year. The rest of the nitrogen is presumably fixed in the soil by the agency of microorganisms.

The first organisms to be studied in connection with the fixation of atmospheric nitrogen were the bacteria forming nodules on the roots of leguminous plants; it was then believed that only those organisms that live symbiotically on the roots of the plants are able, during this process of symbiosis, to transform the gaseous nitrogen of the atmosphere into combined forms.<sup>1</sup> But, in addition to these bacteria, the soil harbors other organisms, which are non-symbiotic and capable of fixing gaseous nitrogen in the presence of a proper source of energy. Berthelot<sup>2</sup> suggested that the fixation of atmospheric nitrogen is well distributed

<sup>1</sup> Beijerinck, M. W. Bot. Ztg. 46: 725-735, 741-750, 758-771, 782-790, 797-803. 1888.

<sup>2</sup> Berthelot, M. Ann. chim. Phys. 13: 5-14, 15-73, 74-78, 78-92, 93-119. 1888. Bull. Soc. Chim. III, 11: 781-783. 1894; Chimie végétale et agricole. Paris, 1899.

among soil microorganisms. However, Winogradsky<sup>3</sup> demonstrated in 1893 that this property is limited to certain specific bacteria. The mere growth of an organism on nitrogen-free media is no indication at all that it is capable of obtaining its nitrogen from the gaseous form of the atmosphere. An organism is considered as unable to fix nitrogen, unless an actual increase in combined nitrogen has been demonstrated by chemical analysis. Beijerinck<sup>4</sup> later established that the number of bacteria in the soil capable of fixing nitrogen is much larger than suspected by Winogradsky.

The first non-symbiotic nitrogen-fixing organism was isolated by Winogradsky in 1893.<sup>5</sup> *Clostridium pastorianum*, an anaerobic organism, belonging to the group of butyric acid bacteria, was found capable of bringing about an increase in the amount of combined nitrogen in the medium, in the presence of an available source of energy. Caron<sup>6</sup> soon (1895) isolated a spore-forming organism, *Bac. ellenbachensis*  $\alpha$ , closely related to *Bac. mycoides* and *Bac. megatherium*, to which he ascribed the property of fixing nitrogen. This claim was confirmed by Stoklasa<sup>7</sup> who reported appreciable gains of combined nitrogen by this organism. The idea then originated to utilize this bacterium for soil inoculation and a special preparation "alinit," in a powdered form, was placed on the market. This proved to be a failure, but it aroused great interest and expectations among the farmers. Actually this organism was later found to be unable to fix any nitrogen.<sup>8</sup>

The most important contribution to the subject of non-symbiotic nitrogen-fixing bacteria, next to Winogradsky's work, was the isolation of the aerobic *Azotobacter chroococcum* and *Azotobacter agile* by Beijerinck.<sup>4</sup> In addition to the *Azotobacter* group, Beijerinck and Van Delden<sup>9</sup> also found that various members of the genus *Granulobacter*

<sup>3</sup> Winogradsky, S. Compt. Rend. Acad. Sci. 116: 1385-1388. 1893; 118: 353-355. 1894; Ann. Sci. Biol. 3: 297-352. 1894-5; Centrbl. Bakt. II, 9: 43-54, 107-112. 1902.

<sup>4</sup> Beijerinck, M. W. Centrbl. Bakt. II, 7: 561-582. 1901.

<sup>5</sup> See Ref.<sup>3</sup>

<sup>6</sup> Caron, A. Landw. Vers. Sta. 45: 401-418. 1895.

<sup>7</sup> Stoklasa, J. Landw. Jahrb. 24: 827-863. 1893; Centrbl. Bakt., II, 4: 39, 78, 119, 284, 507, 535. 1898; 5: 350-359. 1899; 7: 257-270. 1901.

<sup>8</sup> Stutzer, A., and Hartleb, R. Centrbl. Bakt., II, 4: 31-39, 73-77. 1898; Krüger, W., and Schneidewind, W. Landw. Jahrb. 28: 579-591. 1899; see also Beijerinck, M. W. Arch. Neerl. Sci. Exact. Nat., Ser. II, 8: VIII-XXXVI. 1904.

<sup>9</sup> Beijerinck, M. W., and Van Delden, A. Centrbl. Bakt., II, 9: 3-43. 1902.

are capable of fixing nitrogen. A number of other bacteria, commonly found in the soil, are able to fix small amounts of nitrogen on artificial culture media, especially when freshly isolated from the soil.<sup>10</sup>

Algae and fungi do not fix any atmospheric nitrogen, with certain possible exceptions. Symbiosis is claimed for the nodules formed by bacteria on the roots of certain non-leguminous plants, like *Alnus*, *Eleagnus*, *Myrica*, *Coriaria*, *Ceanothus*. Symbiosis between bacteria and the leaves of certain plants was observed in the case of *Pavetta*,<sup>11</sup> *Ardisia*,<sup>12</sup> *Kraussia*.<sup>13</sup> Knots are formed at the place of penetration of the microbe into the tissues of the plant. According to Faber, the bacteria bringing about these formations can also fix nitrogen when not working symbiotically with plants. The amount of nitrogen thus fixed may be so considerable that in India *Pavetta* plants are used as green manure.

Direct nitrogen fixation by higher plants was first suggested by the early chemists, Priestly and Ingenhouse. Positive results were reported also by some recent workers.<sup>14</sup> However, the work of investigators, like Boussingault,<sup>15</sup> Lawes, Gilbert and Pugh,<sup>16</sup> as well as the more recent work of Molliard<sup>17</sup> and numerous others, definitely point to the fact that non-leguminous plants are unable to fix any atmospheric nitrogen.

*Classification of nitrogen-fixing bacteria.* The nitrogen-fixing bacteria require organic compounds of carbon for structural and energy purposes. These organisms can be classified on the basis of their ability to utilize energy in a non-symbiotic manner or obtain it from the growing plant, with which they live symbiotically. None of these organisms are obligate, since they can also obtain their nitrogen from organic or inorganic nitrogen compounds.

<sup>10</sup> For the distribution of nitrogen-fixing bacteria in plankton and sea water, see Keutner, J. *Wiss. Meerunters.* Kiel, N. F. 8: 28. 1904 (*Centrbl. Bakt.* II, 13: 554. 1904); Nakano, H. *Jour. Coll. Sci. Imp. Univ. Tokyo*, 40: 66. 1917.

<sup>11</sup> Faber, F. C. *Jahrb. wiss. Bot.* 51: 285-375. 1912; 54: 243-264. 1914.

<sup>12</sup> Miehe, H. *Ber. deut. bot. Ges.*, 29: 156-157. 1911; 34: 576-580. 1916. *Jahrb. wiss. Bot.* 58: 29. 1917.

<sup>13</sup> Georgevitch. *Bull. Bot. Garden Kew*, 1916, p. 105.

<sup>14</sup> Mameli, E., and Pollacci, G. *Atti. Inst. Bot. Pavia*, Ser. 2, 15: 159-257. 1911; *Centrbl. Bakt.* II, 32: 257. 1912; Lipman, C. B., and Taylor, J. K. *Science*, 56: 605-606. 1922; *J. Frankl. Inst.* 1924, 475-506.

<sup>15</sup> Boussingault, J. B. *Ann. Chim. Phys.* (3) 43: 149-223. 1855.

<sup>16</sup> Lawes, J. B., Gilbert, J. H., and Pugh, E. *Phil. Trans. Roy. Soc. London*, 151: 431-577. 1861; *Rothamsted Mem.* 1: No. 1; 3: No. 1.

<sup>17</sup> Molliard, M. *Rev. Gen. Bot.* 28: 225-250. 1916.

## I. Non-symbiotic nitrogen fixing bacteria:

## 1. Anaerobic bacteria:

- (a) *Clostridium pastorianum*,
- (b) *Bac. saccharobutyricus*,
- (c) *Plectridium* group,
- (d) *Granulobacter* group,

## 2. Aerobic bacteria:

- (a) *Azotobacter* group,
- (b) *Radiobacter* group,
- (c) *Bact. pneumoniae*, *Bact. aerogenes*, and other non-spore forming bacteria,
- (d) *Bac. asterosporus* group and other spore-forming bacteria.

## II. Symbiotic nitrogen-fixing bacteria:

- 1. Bacteria living in the roots of leguminous plants,
- 2. Bacteria living on and in the roots of non-leguminous plants,
- 3. Bacteria living in the leaves of certain plants.

*Isolation of anaerobic bacteria.* For the isolation of bacteria capable of fixing atmospheric nitrogen, Winogradsky<sup>18</sup> used a solution, free from all traces of combined nitrogen, of the following composition:

Distilled water.....	1000 cc.	NaCl.....	0.01 gram
Glucose.....	20.0 grams	FeSO <sub>4</sub> and MnSO <sub>4</sub> ....	Traces
K <sub>2</sub> HPO <sub>4</sub> .....	1.0 gram	CaCO <sub>3</sub> .....	30 grams
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.5 gram		

One hundred cubic centimeters of this medium is placed in a flask and 4 grams of chalk added. The medium is sterilized at 106° to 110° for 30 to 45 minutes. A small quantity of soil, preferably first pasteurized is used for inoculation. After a few days' incubation at 25° to 30°, the surface of the liquid becomes covered with a thin pellicle of aerobic bacteria; gas bubbles are formed abundantly, an indication of butyric acid fermentation. Gas formation begins from the lump of soil and spreads all over the flask, so that the whole surface is soon covered with gas, while the chalk is lumped together by bacterial slime. The culture gives off an odor of butyric acid and its esters. On examining the culture microscopically, it is found that the bacteria in the film and in the residue are not alike. The film contains an aerobic organism, while the residue carries a number of the characteristic cells of the *Clostridium*. Transfers are made, by inoculating a piece of chalk from the bottom of the flask into fresh lots of media. When the culture is sufficiently enriched in *Clostridia* (after three to four transfers), attempts are made at isolation of pure cultures. Pieces of potato smeared with chalk are placed in Petri dishes and sterilized. These are inoculated with spore material and incubated, under anaerobic conditions, at 30° to 35°, in a partial vacuum or hydrogen atmosphere. After 5 to 7 days, there appear upon the surface of the potato elevated, rounded, yellowish colonies filled with gas bubbles. On opening the apparatus, the colonies can be examined for *Clostridia* and transfers made into

<sup>18</sup> Omeliansky, W. L., and Solounskoff, M. Arch. Sci. Biol. 18: 1-24. 1915.

liquid media or fresh potato cultures. The organism often occurs on the potato in involution forms, which temporarily lose the capacity of spore formation. The culture can be grown under aerobic conditions in the presence of an aerobic non-spore forming organism, such as *Bact. fluorescens* or *Azot. chroococcum*. When a pure culture is wanted, the culture is pasteurized (at 75° for 10 minutes), whereby the non-spore forming aerobe is killed and the spore-forming *Clostridium* is obtained pure.<sup>18</sup>

Bredemann<sup>19</sup> used a solid medium of the following composition:

Glucose.....	1.0 gram	{ Agar..... 1.6 grams or
Witte peptone.....	1.2 grams	
Liebig's meat extract..	0.8 gram	{ Gelatin..... 20.0 grams
NaCl.....	0.2 gram	{ Distilled water..... 100 cc.
		{ Reaction slightly alkaline

The various butyric acid bacteria, including the *Cl. pastorianum*, grow very well on this medium, which can be used both for the isolation and cultivation of the organisms.

<sup>19</sup> Bredemann, G. Centrbl. Bakt., II, 22: 44-89. 1908; 23: 385-568. 1909; Ber. deut. bot. Gesell. 26: 362, 795. 1908.

#### PLATE IV

##### NON-SYMBIOTIC NITROGEN FIXING BACTERIA

27. *Clostridium pastorianum*, stage preceding spore-formation; stained with gentian violet, × 660 (from Omeliansky and Solounskoff).

28. *Clostridium pastorianum*, spore-formation, × 660 (after Winogradsky and Omeliansky).

29. *Clostridium pastorianum*, large involution forms; dark color due to coloration of the glycogen with iodine (after Bredemann and Omeliansky).

30. *Azotobacter chroococcum*, young culture (from Krzemieniewski).

31. *Az. chroococcum*, resting forms of sarcina type (from Krzemieniewski).

32. *Az. chroococcum*, thread forms, individual cells undergoing division (from Krzemieniewski).

33. *Az. chroococcum*, growth on agar, showing the darkening of the culture (from Krzemieniewski).

34. *Az. agile*, grown on phosphate-glucose agar, 2 days old, × 660 (from Beijerinck).

35. *Az. agile*, showing flagella, stained by method of Zettnow, × 660 (from Beijerinck).

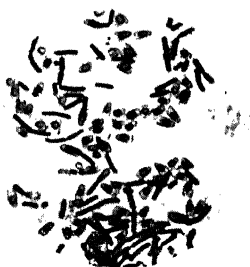
36. *Bac. asterosporus*, 1-5, showing different stages of development and spore formation; 6, spore with folded envelope; 7, cross section of spore (after Bredemann and Omeliansky).

37. *Az. vinelandii* (from Lipman).

38. *Bac. malabarensis*: A, culture on meat extract agar; B, on soil infusion-mannitol agar; C, soil infusion mannitol solution (from Löhnis and Pillai).



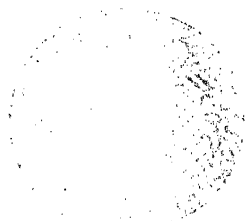
27



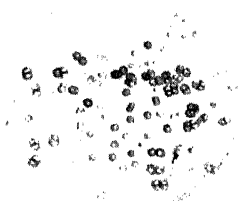
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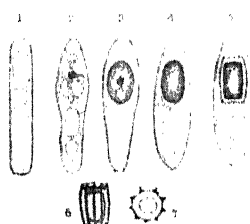
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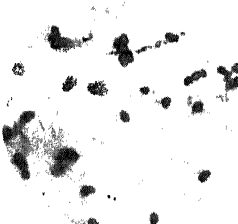
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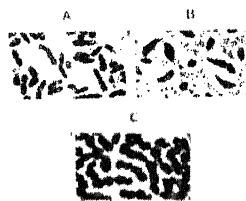
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*Morphology of the anaerobic bacteria.* *Cl. pastorianum* is an actively motile gram-positive rod, occurring singly. The straight cylindrical rods, with rounded ends, are, when young, 1.5 to 2 $\mu$  long by 1.2 to 1.3 $\mu$  thick. They may reach a size of 2 to 6 by 0.8 to 1.3 $\mu$ . The protoplasm is homogeneous, stains well with aniline dyes and gives a yellow color with iodine; older cells become spindle-shaped, coloring violet-brown with iodine. The size of the cells is greatly influenced by the composition of the medium. Both young and old cells are motile, by peritrichous flagella. When spore formation takes place, the rods change into short, thick cells greatly swollen in center, with a diameter twice, or more than twice as great as the original. The cell membrane becomes sharply contoured and surrounds an hyaline substance which encloses the spore. The contents of the cell become granulated, stain with aniline dyes only with difficulty and color violet with iodine. The spore is formed in one end of the enlarged part of the cell and stains well with aniline dyes, but not with iodine; methylene blue stains the spore dark blue and the protoplasm light blue. By the swelling of the hyaline substance, the mother cell bursts open in one end: the ripe spore, 1.6 by 1.3 $\mu$ , is now found to lie in a rounded three cornered spore-capsule. It is characteristic of the species for the capsule to adhere to the spore for a considerable period of time. Under favorable conditions, as on fresh media, the spores swell up and the spore envelope breaks. The spore-germination takes place at one pole towards the open end of the capsule. The young cell soon divides, while the old shell may remain in the liquid for a long time. The cell also produces various involution forms, as long threads irregularly swollen and often carrying a spore at one end. Glycogen and granules accumulate in the cells of the organisms just previous to spore formation.

Bredemann included all the butyric acid bacteria in one species, under the name of *Bac. amylobacter* A. M. et Bred., since the various characteristics, such as size and shape of organism, motility, character of growth on various media, liquefaction of gelatin, carbon sources, products of metabolism, deposition of amylaceous material, were all found to be variable characteristics. Omeliansky,<sup>20</sup> however, did not agree with the grouping of *Clostridium*, *Granulobacter*, *Bac. orthobutylicus* and other butyric acid and butyl alcohol forming bacteria together, and suggested that some of the characters are sufficiently constant to be of value in classification.

The nitrogen-fixing capacity is well distributed among the anaerobic butyric acid bacteria, to a varying extent, however. McCoy, Higby and Fred<sup>21</sup> divided this group into 4 types: 1. *Cl. pastorianum*, or the non-starch fermenting type of Clostridia; 2. *Bac. saccharobutyricus* type of starch fermenting Clostridia and occasional Plectridia; 3. starch-

<sup>20</sup> Omeliansky, W. L. Arch. Sci. Biol., 20: 24-49. 1916.

<sup>21</sup> McCoy, E., Higby, W. M. and Fred, E. B. Centrbl. Bakt., II, 76: 314-320. 1928.



fermenting *Plectridium*, differing from the Plectridia of the previous group by forming long, slender, often curved rods, with thick oval spores at their extreme ends, being also more proteolytic and less fermentative in nature; 4. Butyl-alcohol forming type, morphologically related to the second group of starch fermenting Clostridia. Representatives of the last type fix the least amount of nitrogen, while members of the first two types fix 2.0 to 7.1 mg. of nitrogen per gram of glucose consumed.

*Distribution of anaerobic nitrogen-fixing bacteria in the soil.* Winogradsky has already demonstrated the wide occurrence of *Clostridium*, which he isolated from every soil sample taken in St. Petersburg and in Paris. A larger form was found in southern Russia. Omeliansky demonstrated the presence of this organism in practically all Russian soils; the various strains found in different localities varied greatly in their morphology. Similar results were obtained for German soils.<sup>22</sup> An examination of 152 different soil samples taken in different parts of the world indicated the presence of anaerobic nitrogen-fixing organisms (*Bac. amylobacter*) in 137 cases, including surface soils and subsoils, cultivated and virgin soils, and only irregularly in acid peat soils.<sup>23</sup> Its occurrence in Vesuvian soils has also been established.<sup>24</sup>

The number of nitrogen-fixing Clostridia in the soil was found to be over 100,000 per gram, or much more abundantly than *Azotobacter*.<sup>25</sup> This led various investigators to conclude that the Clostridium rather than Azotobacter is the most important group of non-symbiotic nitrogen-fixing bacteria. Dügge<sup>26</sup> found 100 to 1,000,000 anaerobic and 0 to 100,000 aerobic nitrogen-fixing bacteria per gram of soil. Plots receiving sodium nitrate as a source of nitrogen contained 10,600 to 12,000 cells of Clostridium and 4,900 to 6,300 Azotobacter cells; plots receiving no nitrogen, but potassium and phosphorus fertilizers, contained 1,120,000 Clostridium and 98,700 Azotobacter cells per gram of soil. If one keeps in mind the fact that out of a hundred living cells of *Cl. pastorianum* only very few develop into colonies or give positive growth on artificial culture media, the abundance of this group of bacteria in soil is certainly very extensive. *Cl. pastorianum* is also found

<sup>22</sup> Freudenreich, E. v. Centrbl. Bakt., II, 10: 514-522. 1903.

<sup>23</sup> Haselhoff, E. and Bredemann, G. Landw. Jahrb. 35: 381-414. 1906.

<sup>24</sup> Riccardo, S. Ann. R. Sc. Sup. Agr. Portici, 18: 1-50. 1923.

<sup>25</sup> Truffaut, G. and Bezssonoff, N. Compt. Rend. Acad. Sci. 172: 1319-1322. 1921.

<sup>26</sup> Dügge, 1921 (p. 35).

in soils which are much more acid than the limiting reaction for the growth of *Azotobacter*.<sup>27</sup>

*Physiology of anaerobic nitrogen-fixing bacteria.* Winogradsky found *Cl. pastorianum* to be an obligate anaerobic form which can develop under aerobic conditions only in the presence of aerobic bacteria. However, the facultative aerobic *Cl. americanum* isolated by Pringsheim<sup>28</sup> was very similar in morphology to the other Clostridia, but was capable of fixing larger quantities of nitrogen, perhaps due to its aerobic nature. Bredemann, after examining a large number of Clostridia, came to the conclusion that they are all capable of developing more or less even in the presence of air and fix nitrogen. The presence of 30 mgm. of oxygen per 1 liter of air will still allow spore germination.

The optimum temperature for the development of *Cl. pastorianum* is 28° to 30°C. The spores are not destroyed at 75° even at the end of 15 hours; at 100°C. the spores are destroyed in five minutes. The optimum reaction is pH 6.9 to 7.3, but the organism still develops well at pH 5.7. It can be found in soils even with a pH of 5.0 to 5.2. It can withstand a greater acidity than proteolytic anaerobes like *Bac. putrificus*, from which it can be thus freed.<sup>29</sup> The addition of CaCO<sub>3</sub> to the glucose medium has a favorable effect, in neutralizing the acids formed; MgCO<sub>3</sub> is less favorable. *Cl. pastorianum* can thus withstand a greater acidity than *Azotobacter*, whose limit is pH 6.0. In acid soils (more acid than pH 6.0) *Azotobacter* is inactive while the butyric acid bacteria may still be very abundant.

*Cl. pastorianum* utilizes glucose, levulose, lactose, sucrose, galactose, maltose, raffinose, dextrin, inulin, glycerol, mannitol and lactates. Winogradsky found that the nitrogen source greatly influences the nature of the carbon sources that can be utilized. The greater the concentration of sugar the lower is its economic utilization, 3.2 mgm. nitrogen being fixed per gram of glucose in 0.5 per cent solution, 2 mgm., in 2 per cent solution and 1.2 mgm. in 4 per cent solution.

In the presence of combined nitrogen, nitrogen fixation decreases and comes to a standstill, when the solution contains more than six parts of combined nitrogen in one thousand parts of solution. Omeliansky, however, observed some fixation even with a concentration of 16 parts of combined nitrogen in that quantity of medium.

<sup>27</sup> Omeliansky, W. L. Monogr. No. 5, Russian Acad. Sci. 1923; Truffaut, G. and Bezssonoff, N. Compt. Rend. Acad. Sci. 181: 165-167. 1925.

<sup>28</sup> Pringsheim, H. Centrbl. Bakt. II, 16: 795-800. 1906; 20: 248-256. 1907; 21: 673. 1908; 23: 300. 1909; 24: 488-496. 1909; 36: 468-472. 1913; 40: 21-23. 1914.

<sup>29</sup> Dorner, 1924 (p. 172).

When freshly isolated, the *Clostridium* fixes more nitrogen than when cultivated for a long time in artificial media. The culture can be invigorated by growing it in Winogradsky's liquid medium, to which enough ammonium sulfate is added so as to offer the organism less nitrogen than is needed for the complete decomposition of the sugar. By transferring from this culture, when gas formation ceases, normal growth and nitrogen fixation is obtained. Bredemann<sup>30</sup> invigorated the culture by passing it through soil.

A fertile garden soil is dried, sieved and placed in a flask to a height of 5 cm. The soil is moistened with water and sterilized in an autoclave for 45 minutes at 150°C. When the soil is found to be sterile, it is inoculated with an emulsion of a fresh growth of the weakened culture grown on agar. The flasks are allowed to incubate, one at room temperature under aerobic conditions and one at 28° under anaerobic conditions, for one month, then at room temperature under aerobic conditions. The soil has all dried out by this time. When two grams of it is inoculated into sterile liquid medium, active growth and gas formation begins in 12 hours, reaching a maximum in 36 hours.

This invigorated culture coagulated milk with gas formation; most strains, however, do not grow upon milk. Gelatin was not liquified and casein was not decomposed. Ammonia was not formed from peptone, while nitrates were not reduced.

*Non-symbiotic nitrogen-fixing aerobic bacteria.* When a simple medium containing tap water, 0.02 per cent  $K_2HPO_4$  and glucose as a source of carbon is inoculated with soil and incubated in the dark, *Cl. pastorianum*, together with other bacteria, is obtained. When the glucose is replaced by mannitol (2 per cent) or by propionate of potassium or sodium, another large organism predominates; Beijerinck called this organism *Azotobacter chroococcum* and found it in all soils and manures examined.<sup>31</sup> On repeated transfer to fresh lots of sterile media, the organism was gradually purified from the majority of contaminating forms and finally isolated on mannitol agar. In addition to the above simple medium several other media can be used successfully for the isolation of *Azotobacter*:

1. Beijerinck's medium:

Tap water.....	1000 cc.
Mannitol.....	20 grams
$K_2HPO_4$ .....	0.2 gram

<sup>30</sup> Bredemann, G. Centrbl. Bakt. II, 23: 41-47, 385-568. 1909.

<sup>31</sup> Beijerinck, 1901 (p. 101).

The mannitol can be replaced by dextrin, glycerol, calcium malate (0.5 per cent) and other salts of organic acids. The water may be replaced by soil extract.<sup>32</sup>

2. Lipman's solution:<sup>33</sup>

Distilled water.....	1000 cc.	MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.2 gram
Mannitol.....	15 grams	CaCl <sub>2</sub> .....	0.02 gram
K <sub>2</sub> HPO <sub>4</sub> .....	0.2 gram	FeCl <sub>3</sub> 1 drop of 10 per cent solution	

The substances are dissolved, and enough 10 per cent NaOH is added to make faintly pink to phenolphthalein.

3. Ashby's solution:<sup>34</sup>

Distilled water.....	1000 cc.	NaCl.....	0.2 gram
Mannitol.....	10 grams	CaSO <sub>4</sub> ·2H <sub>2</sub> O.....	0.1 gram
KH <sub>2</sub> PO <sub>4</sub> .....	0.2 gram	CaCO <sub>3</sub> .....	5.0 grams
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.2 gram		

Make neutral to phenolphthalein with NaOH solution.

4. Omeliansky's solution:<sup>35</sup>

Distilled water.....	1000 cc.	MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.5 gram
Dextrin.....	20 grams	CaCO <sub>3</sub> .....	10.0 grams
K <sub>2</sub> HPO <sub>4</sub> .....	1.0 gram		

Solid media are prepared by adding 15 or 20 gm. of agar to the above solutions. For the study of pigment formation the last medium is very appropriate. Potato and potato agar, milk, mannitol-nitrate media are also employed for the study of the morphology of the organism.

A small quantity of fertile garden soil, or well limed and manured field soil is used for the inoculation of the sterile liquid medium. After a few days' incubation at 25° to 30°C., a pellicle is formed on the surface of the liquid, at first gray in color, later becoming brownish. A microscopic examination of the pellicle shows the presence of the typical *Azotobacter* cells with large slimy capsules. A part of the pellicle is then transferred into a fresh flask with sterile medium. After several transfers, the culture is sufficiently enriched in *Azotobacter* so that one can proceed to isolate it on agar media. A loopful of material from a young culture, in which no heavy pellicle has as yet been formed, is diluted in a few cubic centimeters of sterile water to separate the *Azotobacter* cells. A second and third dilution is made and the surfaces of a series of Petri dishes, in which liquefied mannitol agar has been placed and allowed to cool, are then streaked out with the suspensions of the various dilutions.

<sup>32</sup> Löhnis, F. Centrbl. Bakt. II, 14: 582-604, 713-723. 1905; Landwirtschaftlich-bakteriologisches Praktikum. 1911. p. 131.

<sup>33</sup> Lipman, J. G. N. J. Agr. Exp. Sta. 25th Ann. Rpt. 1904, 237-289.

<sup>34</sup> Ashby, S. F. Jour. Agr. Sci. 2: 35-51. 1907.

<sup>35</sup> Omeliansky, W. L. and Sseverowa, O. P. Centrbl. Bakt. II, 29: 643-650. 1911.

At 25°C., pale, rounded, raised colonies are formed in a few days. In addition to these, transparent raised colonies of *Radiobacter* are also found. The *Azotobacter* colonies are carefully selected by microscopic examination and are transferred into sterile liquid media. These precautions are very important so as not to have the culture contaminated.

When a silica gel plate, to which minerals and mannitol have been added, is inoculated with small particles of soil a nearly pure culture of *Azotobacter* can be readily obtained, as shown by Winogradsky.<sup>36</sup>

*Description of species of Azotobacter.* *Azotobacter* is a bacterium, 4 to 6 $\mu$  broad, forming, when young, diplococci or short rods, with hyaline contents, often containing a vacuole and forming a slimy wall. Various involution forms, as threads reaching 60 to 80 $\mu$  in length, may be produced. Formation of nuclei has been demonstrated by Prazmowski. *Azotobacter* is aerobic and readily killed on heating. Beijerinck, Lipman and Jones<sup>37</sup> denied the existence of spores in *Azotobacter*. According to Ashby, Fischer and Krzemieniewski, spore formation is positive, since it can resist drying for a long period of time. Prazmowski<sup>38</sup> demonstrated that spore formation takes place under normal conditions, with sufficient aeration and in the presence of humates. The spores are apparently not endospores but arthrospores.<sup>39</sup>

*Azotobacter chroococcum* Beij. is universally distributed in soils having a reaction above pH 6.0. It produces a crude floating membrane on tap water containing 2 per cent mannitol and 0.02 per cent  $K_2HPO_4$  inoculated with garden soil. Only occasional individuals in young cultures are motile by means of a single flagellum. The size of the cell is reported by investigators differently, varying from 3 to 4 by 5 to 6 $\mu$  and even 3 to 4 by 9 to 12 $\mu$  to 2 to 3 by 3 to 4 $\mu$  (limits 1.5 to 7 $\mu$ ) and even 1 to 2 $\mu$  for cocci and 1.5 to 2 by 3 to 4 $\mu$  for rods.<sup>40</sup> Old membranes consist of micrococci of various sizes united to form sarcina-like masses, possessing mucilaginous walls. The older cultures are frequently brown or black. This organism oxidizes numerous carbon compounds to  $CO_2$  and  $H_2O$ .

*Azotobacter agile* Beij. was isolated from the canal waters of Delft; the crude and pure cultures are obtained by previous methods. The organism is very motile by means of a bundle of polar flagella. The cells are large, transparent, resem-

<sup>36</sup> Winogradsky, 1925 (p. 8).

<sup>37</sup> Jones, D. H. Centrbl. Bakt., II, 38: 14-21. 1913. Jour. Bact. 5: 325. 1920.

<sup>38</sup> Prazmowski, A. Centrbl. Bakt. II, 33: 292-305. 1912; 37: 299. 1913; Ariz. Akad. Krakau. Math.-Naturw. Kl. (B), 1912, 855-950. (Centrbl. Bakt. II, 37: 299-301. 1913.

<sup>39</sup> Löhnis and Smith, 1916 (p. 56); Mulvania. Science, 42: 463. 1915.

<sup>40</sup> Bonazzi, A. Jour. Agr. Res., 4: 225-241. 1915.

bling monads, often with a clearly discernible cell wall, protoplasm, nucleus, granules and vacuoles. In the presence of salts of organic acids, it produces a green or red diffusible pigment.

*Azotobacter vinelandii* was isolated by Lipman<sup>41</sup> from a New Jersey soil in 1903. In four days it forms on mannitol agar colonies 4 mm. in diameter. These are round, raised, concentric and semi-transparent, with denser whitish centers; the deep colonies are white, small, hardly more than 1 mm. in diameter, elliptical to spindle-shaped. A white thick membrane is formed in the mannitol solution. When undisturbed, the liquid culture shows the formation of a bright yellow pigment, concentrated near the surface and gradually diffusing through the liquid. In older cultures the pigment gradually diffuses throughout the medium and becomes darker, until in old cultures it may become a yellowish red. At the same time the bacterial mass may also become darker. A considerable number of forms, ranging from large rods with rounded ends to spherical organisms, are found in mannitol cultures. Most of the organisms are actively motile, showing progressive and at times rotatory motility. As the culture grows older, the number of shorter rods increases, and the cells begin to accumulate and store up fat, which appears in small globules throughout the bacterial body and gives it a granular appearance. Various involution forms are produced in meat extract bouillon. A temperature of 85°C. for five minutes is sufficient to destroy all the cells. The organism stains readily with carbol fuchsin, with aqueous solutions of gentian violet, methyl violet or fuchsin and Löffler's methylene blue. The bright yellow pigment produced in mannitol solution is favored by greater surface of medium (oxygen need), is soluble in alcohol and decolorized by weak acids. The organism is very active in the fixation of atmospheric nitrogen.

*Azotobacter beijerinckii* was isolated by Lipman in 1904. It forms pure white, moist, soft, irregularly round, netted colonies. In mannitol solution, it forms a turbidity, with white circular dots on surface and walls, gradually settling to the bottom. The cells are large, almost spherical, occurring singly, or in chains of two or more. This organism is much larger than *A. chroococcum* and *A. vinelandii* and does not show any motility. On solid media, a yellowish pigment is formed. *Azotobacter vitreum* was isolated by Löhnis and Westermann.<sup>42</sup> It forms only round cells, is non-motile, grows as transparent, moist colonies on solid media, without any pigment. Prazmowski expressed his doubts whether this organism belongs to the genus *Azotobacter*.

*Distribution of Azotobacter in the soil.* *Azotobacter* is of universal occurrence in the soil.<sup>43</sup> Out of one hundred and five soil samples examined, Burri<sup>44</sup> found *Azotobacter* missing in thirty-four cases, chiefly in heavy clay soils. Jones and Murdoch<sup>45</sup> found *Azotobacter* in nine

<sup>41</sup> Lipman, J. G. N. J. Agr. Exp. Sta. Ann. Rpt. 24: 217-285. 1903; 25: 237-289, 1904; 26: 254. 1905; 29: 137. 1908.

<sup>42</sup> Löhnis, F. and Westermann, T. Centrbl. Bakt. II, 22: 234-254. 1908.

<sup>43</sup> Beijerinck and Van Delden. 1902 (p. 101).

<sup>44</sup> Burri, R. Schweiz. Ztschr. Forstwesen. 55: 89. 1904.

<sup>45</sup> Jones, D. H. and Murdoch, F. G. Soil Sci. 8: 259-267. 1919.

out of seventeen soil types examined and in twenty-two out of twenty-nine soil samples representing nine types. Eighteen was the maximum number of *Azotobacter* cells found per one gram of soil.

The absence of *Azotobacter* in the soil is probably due, in the majority of cases, to the soil reaction, since this organism cannot develop in a soil having an acidity greater than pH 6.0. As soon as the reaction of the soil is adjusted by means of lime so that the pH becomes greater than 6.0, an *Azotobacter* flora will develop.<sup>46</sup>

*Azotobacter* is widely represented in the soil by various forms, so that Löhnis and Westermann counted in 1908 as many as twenty-one different types. These were believed, however, to represent only four species. Lipman and Burgess isolated in 1915 a number of forms from American and foreign soils including several new species. The most common type, however, was in both cases *A. chroococcum*. As a result of a study of a large number of soils, Lipman and Burgess<sup>47</sup> came to the conclusion that, with a proper supply of energy producing materials, all agricultural soils may be made to fix atmospheric nitrogen when inoculated into a properly constituted mannitol solution; however, only a fraction of these soils (one-third) contain *Azotobacter*. Those soils that contain *Azotobacter* have a more vigorous nitrogen fixing power. As much as 10 mgm. of nitrogen are fixed per gram of mannitol in solution and 12.6 mgm. in soil by pure cultures. Gainey compared the nitrogen fixing capacity of a large number of soils by inoculating 50 cc. of a 2 per cent mannitol-mineral salt solution. The average amount of nitrogen fixed in 3 weeks by all the soils was 6.36 mgm. 174 soils containing *Azotobacter* fixed on the average 8.30 mgm., while 193 soils not containing *Azotobacter* fixed only 4.61 mgm.

*Azotobacter* was demonstrated in most Javan soils, in all soils in India, in a half of Polish soils and in about 33 per cent of the cultivated soils in Japan.<sup>48</sup> The wide distribution of *Azotobacter* in Russian, Utah and Danish soils has also been pointed out.<sup>49</sup> It is almost com-

<sup>46</sup> Christensen, H. R. Intern. Mitt. Bodenk. 13: H. 3-4. 1923; Gainey, P. L. Jour. Agr. Res. 24: 289-296, 759-767, 907-938. 1923; Waksman, S. A. Science, 48: 653. 1918.

<sup>47</sup> Lipman, C. B. and Burgess, P. A. Centrbl. Bakt. II, 44: 481-511. 1915.

<sup>48</sup> Groenewege, J. Arch. Suikerind. 21: 790-793. 1913; Hutchinson, C. M. Rpt. Agr. Res. Inst. Pusa. 1911-12, 85-90; Mem. India Agr. Exp. Sta. Bact. Ser. 1: 98-112. 1915; Ziemiańska, J. Roczn. Nauk Roln. 10: 1-78. 1923; Yamagata, U. and Itano, A. Jour. Bact. 8: 521-531. 1923.

<sup>49</sup> Greaves, J. E. Soil Sci. 6: 163-217. 1918; Weis, F. and Bornebusch, C. H. Det. Forstlige Forsogs. Denmark, 4: 319-331. 1914.

pletely absent in Finnish soils, even in those that are well buffered and supplied with  $\text{CaCO}_3$ .<sup>50</sup>

*Azotobacter* derived from different localities may vary greatly in the amounts of nitrogen fixed. In humid regions the nitrogen-fixing organisms are confined to the upper few inches of soil. However, in arid regions, they may be quite active to a depth of 3 to 4 feet. *Azotobacter* occurs more frequently in cultivated than in virgin soils. The number of *Azotobacter* in the soil is highest in spring and fall of year and lowest in summer and winter. According to Beijerinck, the number of *Azotobacter* in the soil runs parallel with soil fertility. It is interesting to note that *Azotobacter* is among the first organisms to develop in a newly formed soil, as in the case of Vesuvian soils.<sup>51</sup>

*Morphology and life cycle of Azotobacter.* The form and size of *Azotobacter* cells depend upon the composition of the medium and conditions of cultivation. Increased aeration brings about a lengthening of the forms and greater motility. In the presence of organic colloidal substances, especially those containing nitrogen, as well as aluminum salts, the cells remain in a young condition for a long time; alkali salts and other salts stimulate the maturity of the culture. Prazmowski reported that the dark brown *A. chroococcum* can change into a colorless race, similar to *A. vinelandii* and *A. agile*, and a yellow race similar to *A. beijerinckii*; he concluded, therefore, that the various species described are merely races of one greatly variable species. These results were confirmed by Omeliansky, who found that pigment formation by *Azotobacter* depends entirely on the composition of the medium. However, this is not sufficient to deny the existence of the various species altogether, especially since the genus itself is very variable, and it is difficult to establish the limits for its systematic position. The organism most commonly studied is *A. chroococcum*. It goes through a regular life cycle. Drying stimulates spore formation. The membrane surrounding the cell becomes more compact and thinner while the cell itself changes into a spore.<sup>52</sup> Prazmowski observed spore formation

<sup>50</sup> Brenner, W. Geolog. Kom. Finland. Agr. Geol. Meddl. 20: 1924. Attention should be called to the fact that soils frequently contain bacteria which resemble microscopically *azotobacter* cells, but which are unable to fix nitrogen. This explains the frequently observed difference between the microscopic and silica gel plate methods for demonstrating the abundance of these organisms in soil. Dianova, E. B. and Voroshilova, A. A. Nauch. Agron. Zhur. 7: 259-270. 1930; Centrlel. Bakt. II, 84: 433-452. 1931.

<sup>51</sup> Riccardo, 1923 (p. 106).

<sup>52</sup> Fischer, H. Centrbl. Bakt. II, 14: 33-34. 1905; 15: 235-236. 1906.



also in ordinary media, containing soil extract. Several spores were found to be produced in a cell and these are believed to be responsible for the irregular packet and sarcina forms observed in mature cultures. The latter are formed by simple fission of the cell.<sup>53</sup> The granules arising from the splitting up of the supposed nuclear body may act as gonidia spores.

Löhnis and Smith brought a certain amount of evidence to indicate that the genus *Azotobacter* is characterized by several different cell types:

1. Large non-sporulating, globular, oval, or rod-like cells, with polar or peritrichous flagella.
2. Coccoid cells, the vegetative growth of the regenerative bodies, identical with *Micrococcus*.
3. Dwarfed cell type, the vegetative growth of the gonidia.
4. Irregular, fungoid cells, similar to *Mycobacterium*.
5. Small non-sporulating rods, identical either with *Bact. lactis viscosum* or *Bact. putidum*.
6. Small sporulating rods, identical either with *Bac. fusiformis* or *Bac. pumilus*.
7. Large sporulating cells.

Several types of reproductive organs of *Azotobacter* were recognized:

1. Gonidia, in part filterable, produced in the cell.
2. Regenerative bodies and exospores, either produced by the cells or growing up from the symplasm.
3. Arthrospores formed by fragmentation of the rod-like or fungoid cells.
4. Mycrocysts, globular or oval resting cells.
5. Endospores, produced singly by the rod-like cells in terminal or in central position, or in the form of two or more globular or spindle-shaped sporangia.

In accordance with this theory, gonidia form the basis for the development of the regenerative bodies, arthrospores and endospores, while symplasm formation and regeneration of new cells proceeds with *Azotobacter* as with other bacteria.<sup>54</sup>

Löhnis and Smith claimed that only two species of *Azotobacter* were isolated so far; namely *A. chroococcum* and *A. agile*. *A. beijerinckii* Lipman was looked upon as a variety of *A. chroococcum*, while *A. vinelandii* Lipman and *A. vitreum* Löhnis were considered to be varieties of *A. agile*. Wilke and Niemeyer<sup>55</sup> also established the disintegration

<sup>53</sup> Jones, D. H. Centrbl. Bakt. II, 38: 14-25. 1913; 42: 68-9. 1914; Trans. Roy. Soc. Canada. Ser. 3, 7: 1913, Sect. IV; Jour. Bact. 5: 325-342. 1920.

<sup>54</sup> On the systematic position of *Azotobacter*, the work of Löhnis and Hanzawa should be consulted. Centrbl. Bakt. II, 42: 1-8. 1914.

<sup>55</sup> Wilke, F. Bot. Archiv. 30: 307-343. 1930; Niemeyer, L. Ibid. 7: 347-374. 1924.

of *Azotobacter* cells and formation of an amorphous mass, but they could never demonstrate the rebuilding of vegetative cells from the symplasm, hence they denied entirely the formation of any true symplasm.

*Physiology of Azotobacter.* There is an important difference in the amount of nitrogen fixed by pure cultures of *Azotobacter* and by crude cultures or in the presence of other organisms, in favor of the last. This was first recognized by Beijerinck and Van Delden, who went as far as to state that *A. chroococcum* is incapable of fixing any appreciable quantities of nitrogen when growing in pure culture, but that large amounts of nitrogen are fixed in the presence of the spore bearing *Granulobacter* or non-spore bearing members of the *Bact. aerogenes* group and *Bact. radiobacter*. Members of the *Granulobacter* group were found capable of fixing nitrogen by themselves, this power becoming very pronounced in the presence of *Azotobacter*. On the other hand, Gerlach and Vogel<sup>56</sup> proved conclusively that *A. chroococcum* is able to fix large quantities of atmospheric nitrogen when grown in pure culture, in the presence of salts of organic acids or sugar. This was soon confirmed by others.<sup>57</sup> The presence of other organisms, however, is advantageous to the amount of nitrogen fixed and the rate of fixation, either by using up the waste products or creating otherwise favorable conditions. Young cultures will fix more nitrogen than old cultures; crude cultures more than pure cultures.

A number of hexoses (glucose), pentoses, alcohols (mannitol) and salts of organic acids, such as malate,<sup>58</sup> lactate, butyrate, succinate, can be utilized as sources of energy by *Azotobacter*. The available evidence seems to indicate that celluloses may become available as sources of energy to nitrogen-fixing bacteria only when cellulose decomposing bacteria are present, but not directly,<sup>59</sup> although even this still needs further confirmation. Ammonium salts are utilized more readily than nitrates as sources of nitrogen. Only traces of minerals are required, except phosphates, large quantities of which are needed for the synthesis of the cells. The organism can withstand as much as 10 per cent  $MgSO_4$ . *Azotobacter* resists drying very readily<sup>60</sup> and is

<sup>56</sup> Gerlach, M. and Vogel, I. Centrbl. Bakt., II, 8: 669-674. 1902; 9: 817-821, 881-892. 1902; 10: 636-643. 1903.

<sup>57</sup> Freudenreich, 1903 (p. 106); Lipman, 1903 (p. 111).

<sup>58</sup> Beijerinck, M. W. Centrbl. Bakt. II, 63: 353-359. 1925.

<sup>59</sup> Pringsheim, H. Centrbl. Bakt. II, 23: 300-304. 1909; 26: 222-227. 1910; Biol. Centrbl. 31: 65. 1911; Hutchinson and Clayton, 1918 (p. 190).

<sup>60</sup> Stapp, C. and Ruschmann, G. Arb. Biol. Reichsanst. Land. u. Forstw. 13: 305-368. 1924.

sensitive to high temperature; the cells are destroyed in a few minutes at 55°C.

*Other non-symbiotic nitrogen-fixing bacteria.* In addition to the anaerobic butyric acid bacteria and *Azotobacter*, various other bacteria are capable of fixing appreciable quantities of nitrogen. This is true of *Bact. lactis viscosum*, *Bact. pneumoniae*, *Bact. radiobacter* and *Bact. prodigiosum*.<sup>61</sup> *Bact. aerogenes*, *Bact. pyocyaneum* and a number of other bacteria were also found capable of fixing small amounts of nitrogen, especially when freshly isolated from the soil.<sup>62</sup> Two representatives of the *Bac. mesentericus* group, namely *Bac. malabarensis* and *Bac. danicus*, the first isolated from South Indian rice soil and the second from nodules of *Vicia*, were found capable of fixing nitrogen. *Bact. radicicola*, the legume organism, can live in the soil outside of the nodules; it was at first believed to be capable of fixing small quantities of atmospheric nitrogen on artificial media.<sup>63</sup> More recent investigations<sup>64</sup> seem to question, however, the non-symbiotic nitrogen-fixing capacity of this organism. *Bac. asterosporus* can fix 1 to 3 mgm. nitrogen for every gram of glucose consumed; the organism soon loses this property, but it can regain it on passage through soil.<sup>65</sup> Bondorff described *Planobacillus nitrofigens*, a large rod-shaped, spore-forming bacterium, capable of fixing in three weeks 3.57 mgm. of nitrogen per 100 cc. of solution of a soil extract medium containing 2 per cent of mannitol.<sup>66</sup> A form related to *B. vulgare* was found capable of fixing 1.8 to 4.7 mgm. of nitrogen for one gram of sugar consumed.<sup>67</sup> A nitrogen-fixing organism (*Bac. azophile*) was also isolated from manure.<sup>68</sup> Certain thermophilic bacteria, growing in mixed culture at 61°C., were claimed to be capable of fixing appreciable quantities of nitrogen.<sup>69</sup>

<sup>61</sup> Löhnis, F. Centrbl. Bakt. II, 14: 582-604. 1905. Löhnis, F., and Pillai, N. K. Centrbl. Bakt. II, 19: 87-96. 1907; 20: 781-799. 1908.

<sup>62</sup> Selim, M. Centrbl. Bakt. II, 83: 311-325. 1931. Not all strains of *B. aerogenes* are capable of fixing nitrogen, as shown by Skinner, C. E. Soil Sci. 25: 195-205. 1928.

<sup>63</sup> Mazé, P. Ann. Inst. Past. 11: 44-54. 1898; Löhnis, 1910 (p. xiv); Fred, E. B. Va. Agr. Exp. Sta. Ann. Rpt. 1909-1910, 138-142.

<sup>64</sup> Barthel, Chr. Meddel. Centralanst. Jordbr. Bakt. Avd. 43, 1926. A detailed review of the subject of nitrogen fixation by nodule bacteria in pure culture is given by Hopkins, E. W. Soil Sci. 28: 433-448. 1929; Allison, F. E. Jour. Agr. Res. 39: 893-924. 1929; Löhnis, M. P. Soil Sci. 29: 37-58. 1930.

<sup>65</sup> Bredemann, G. Centrbl. Bakt. II, 22: 44-89. 1908.

<sup>66</sup> Bondorff, K. A. Den. Kgl. Veterinaer og Landboholskr. Aarskr. 1918, 365-369.

<sup>67</sup> Truffaut, G. and Bezssonoff, N. Compt. Rend. Acad. Sci. 175: 544. 1922.

<sup>68</sup> Fulmer, H. L. and Fred, E. B. Jour. Bact. 2: 423-434. 1917.

<sup>69</sup> Pringsheim, H. Centrbl. Bakt. II, 31: 23-27. 1911.

*Azotobacter* may live symbiotically with algae,<sup>70</sup> especially with *Nostoc* and *Anabaena*, as well as with other bacteria.<sup>71</sup> The quantities of nitrogen fixed by this association are frequently quite considerable.<sup>72</sup> The symbiotic action between *Cl. pastorianum* and *Azotobacter*, whereby the second uses up the oxygen making conditions favorable for the former, has been demonstrated.<sup>73</sup> The various acids produced by the former are neutralized by the soil bases and can be utilized by *Azotobacter* as sources of energy; this symbiotic action leads to a maximum economy in the utilization of energy.

According to Winogradsky, the nitrogen fixing bacteria commonly reported to be present in the soil must be divided into two groups: 1. Natural fixing agents, comprising the two classical groups of *Cl. pastorianum* and *Azotobacter*, organisms whose normal functions consist in the fixation of nitrogen. 2. Artificial fixing agents or those organisms which can fix small amounts of nitrogen only under artificial laboratory conditions and which do not seem to take any part in the process of nitrogen-fixation in the soil. Löhnis and others, however, are of the opinion that the property of fixing small quantities of nitrogen is widely distributed among soil microorganisms.

*Symbiotic nitrogen-fixation by nodule bacteria. Historical.* Many centuries before the discovery of the nodule bacteria and before their part in the enrichment of the soil with combined nitrogen, due to their symbiotic action with leguminous plants, had been established, the practical agriculturist came to consider the growth of these plants in the soil equivalent to manuring the soil for the succeeding crop.<sup>74</sup>

In the early part of the 19th century Davy<sup>75</sup> stated that "peas and beans in all instances seem well adapted to prepare the ground for wheat. Peas and beans contain a small quantity of a matter analogous to albumen; but it seems that the azote which forms a constituent part

<sup>70</sup> Reinke, J. Ber. deut. bot. Gesell. 21: 482. 1903; Fischer, H. Centrbl. Bakt. II, 12: 267. 1904; Heinze, B. Centrbl. Bakt. II., 16: 640-653, 703-711. 1906; Landw. Jahrb. 35: 889-910. 1906; Drewes, K. Centrbl. Bakt., II, 76: 88-101. 1928.

<sup>71</sup> Beijerinck and van Delden, 1902 (p. 101).

<sup>72</sup> Hutchinson, C. M. Agr. Res. Inst. Pusa., Sci. Rpts. 1922-23, 43-49.

<sup>73</sup> Omeliansky, 1916 (p. 105).

<sup>74</sup> Use of legumes by Romans is found in the work of Plinius—*Historia naturalis* LVIII; Varro—*De re rustica*. Lib. I, Chap. 23; by ancient Chinese, in the book of F. H. King—*Farmers of forty centuries*. Harcourt, Brace & Co. New York. 1927.

<sup>75</sup> Davy, Sir Humphry. *Elements of agricultural chemistry*. 2nd Ed. 1819.

of this matter is derived from the atmosphere." Boussingault<sup>76</sup> was the first to carry out a series of systematic studies on the nitrogen nutrition of leguminous and cereal plants. He established the fact that, in the cultivation of clover in unmanured soils, there is a definite gain, not only of carbon, hydrogen and oxygen, but also of large quantities of nitrogen; wheat, however, under the same conditions shows no gain or loss in nitrogen. Boussingault definitely expressed his opinion that nitrogen belongs to those elements which leguminous plants (clover, peas) can assimilate from the atmosphere, while cereal plants (wheat, oats) cannot do so. In attempting to repeat these experiments under more carefully controlled conditions, Boussingault ignited the sand (thus killing the bacteria) and found that neither cereals nor legumes were capable of assimilating atmospheric nitrogen.<sup>77</sup>

In an elaborate series of experiments begun in 1857 at the Rothamsted Experimental Station, Lawes, Gilbert and Pugh<sup>78</sup> were so careful to eliminate any possibility of the plants obtaining any combined nitrogen from the atmosphere, that they destroyed the organism fixing the nitrogen symbiotically with leguminous plants; they thus failed to become the discoverers of this symbiotic relationship, since, in the absence of the bacteria, the leguminous plants behaved like the cereals and could not utilize the atmospheric nitrogen. Bretschneider's<sup>79</sup> results obtained in 1861 show that legumes do not fix any nitrogen when the soil is ignited but do so in unignited soil. Schulz-Lupitz<sup>80</sup> grew lupines for fifteen consecutive times, without the application of nitrogen fertilizer and without diminishing yields; cereals following lupines gave much higher yields than on the same land not preceded by the leguminous crop; the nitrogen content of the soil was actually found to increase.

The presence of nodules on the roots of leguminous plants was recorded by Dalechamps in 1586 and by Malpighi<sup>81</sup> in 1679, but they, as well as others, considered them to be root galls. Lachmann<sup>82</sup> observed,

<sup>76</sup> Boussingault. *Compt. Rend. Acad. Sci.* 6: 102-112. 1838; 7: 889-892; *Ann. Chim. et phys.* (2), 67: 1-54. 1838; 69: 353-367. 1838; *Compt. Rend. Acad. Sci.* 38: 580-607. 1854; 39: 601-613.

<sup>77</sup> Ville. *Compt. Rend. Acad. Sci.* 31: 578. 1850; 35: 464-468, 650-654. 1852; 38: 705-709, 723-727. 1854; 43: 143-148. 1856.

<sup>78</sup> Lawes, Gilbert and Pugh, 1861 (p. 102).

<sup>79</sup> Bretschneider. *Jahresber. Agr. Chem.* 4: 123. 1861-1862.

<sup>80</sup> Schultz, L. *Landw. Jahrb.* 10: 777-848. 1881.

<sup>81</sup> Malpighi. *Opera omnia. Anatomia plantarum, Pars II. De gallis.* 1679.

<sup>82</sup> Lachmann. *Landw. Mitt. Zeitschr. K. Lehranstalt. u. Vers. Sta* 1858, p. 37.

in 1858, that motile bacteria cause the formation of the nodules and he suggested that the nodules are the organs of nitrogen fixation. In 1866 Woronin<sup>83</sup> demonstrated that the nodules consist of bacteria, but even he considered these nodules as pathological outgrowths. Eriksson<sup>84</sup> also observed the bacteria-like bodies in the older cells of the nodule tissue; he believed that the infection threads were mold hyphae and were the true cause of nodule formation. Frank<sup>85</sup> established in 1879 that the formation of nodules can be prevented by the sterilization of the soil. Frank's view as well as that of other investigators<sup>86</sup> was that the nodules are caused by outside infection. Hellriegel and Wilfarth<sup>87</sup> finally demonstrated in 1884-1886 that the nodules on the roots of leguminous plants are due to bacterial infection, that this is beneficial, since it is within these nodules where the bacteria fix the atmospheric nitrogen. When nodules were formed, the plants could be grown on artificial soils containing but traces of combined nitrogen, provided the mineral elements necessary for the nutrition of the plant were present. In the absence of nodules, the plants were unable to utilize the atmospheric nitrogen for their growth. When sterilized soil was treated with fresh soil infusion, nodule formation took place and the plants grew normally. The growth of the Gramineae depended, however, on the nitrate content of the soil. These results were soon confirmed by Lawes and Gilbert<sup>88</sup> and others.

The causative organism was isolated in 1888, in pure culture, by Beijerinck, who named it *Bacillus radicicola*. Beijerinck described three stages in the development of the bacterium.

1. The organism is present in the soil in the form of small rods which can penetrate the root hairs of the leguminous plants and from there it is transferred to the "infectious tissue."
2. It changes later into a motile bacillus.
3. It is finally transformed into the bacteroid which functions as the symbiotic organism.

<sup>83</sup> Woronin, M. Ann. Sci. Nat. Bot., ser. 5, 7: 73-86. 1867; Mem. Acad. Imp. Sci. St. Petersburg, (7), 10: 1-13. 1866.

<sup>84</sup> Eriksson. Acta Univ. Lund. 10: 1-30. 1873 (Bot. Ztg. 32: 381-384. 1874).

<sup>85</sup> Frank, B. Bot. Ztg. 37: 377-388, 393-400. 1879.

<sup>86</sup> Ward, M. Phil. Trans. Roy. Soc. London 178, 1887.

<sup>87</sup> Hellriegel, H. Tagebl. Natforsch. Vers. Berlin, 1886, p. 290; Chem. Centrbl. 1886, 781; Landw. Vers. Sta. 33: 464-465. 1886. Hellriegel, H. and Wilfarth, H. Beilageheft Ztschr. Ver. Rübenzuckerind. 1888, 1-234; see also Atwater, W. O. Rpt. Brit. Assn. Adv. Sci. 54: 685. 1884; Amer. Chem. Jour. 6: 365-388. 1885; 8: 398-420. 1886; Conn. (Storrs) Agr. Exp. Sta. Bul. 5, 1889; Ann. Rpt. 1889, 11-51.

<sup>88</sup> Lawes, J. and Gilbert, J. Proc. Roy. Soc. London 47: 85-118. 1890.

The bacterium was soon grown, on artificial culture media, by a number of investigators.<sup>89</sup> The mechanism of root infection by a pure culture of the organism was worked out by Prazmowski in 1889.<sup>90</sup> Schloesing and Laurent<sup>91</sup> demonstrated that the nitrogen is actually obtained by the association of the plant and bacterium in the form of nitrogen gas from the atmosphere. Leguminous plants were grown in sterile glass cylinders containing sterile sand and watered with sterile water. When the composition of the gas in the cylinder was determined, it was found that, while the uninoculated plants showed a gain of only 0.6 mgm. of nitrogen and no nodule formation, inoculated plants

<sup>89</sup> For a review of earlier literature see Voorhees and Lipman, Bul. 194, Office Exp. Sta., U. S. Dept. Agr., 1907. Hiltner, 1904 (p. 123); Löhnis, 1910 (p. xiv); Burrill and Hansen, 1917 (p. 121); Müller, A., and Stapp, C. Arb. Biol. Reichsanst. L. u. Forstw. 14: 455-554. 1925.

<sup>90</sup> Prazmowski, A. Bot. Centrbl. 39: 356-362. 1889; Landw. Vers. Sta. 37: 161-238; 38: 5. 1890.

<sup>91</sup> Schloesing, Th. and Laurent, E. Compt. Rend. Acad. Sci. 111: 750-754, 1890; 113: 776-778, 1059-1060. 1891; 115: 1017. 1892; Ann. Inst. Past. 6: 65-115, 824-840. 1892.

## PLATE V

### SYMBIOTIC NITROGEN FIXING BACTERIA

39. Types of nodules on leguminous and non-leguminous plants: a, *Trifolium pratense*; b, *Soja hispida*; c, *Alnus*; d, *Vicia faba*; e, Cowpea; f, nodules on leaf of *Pavetta indica*. (a, after Omeliansky; b, e, after Albrecht; c, after de Rossi; d, f, after Omeliansky.)

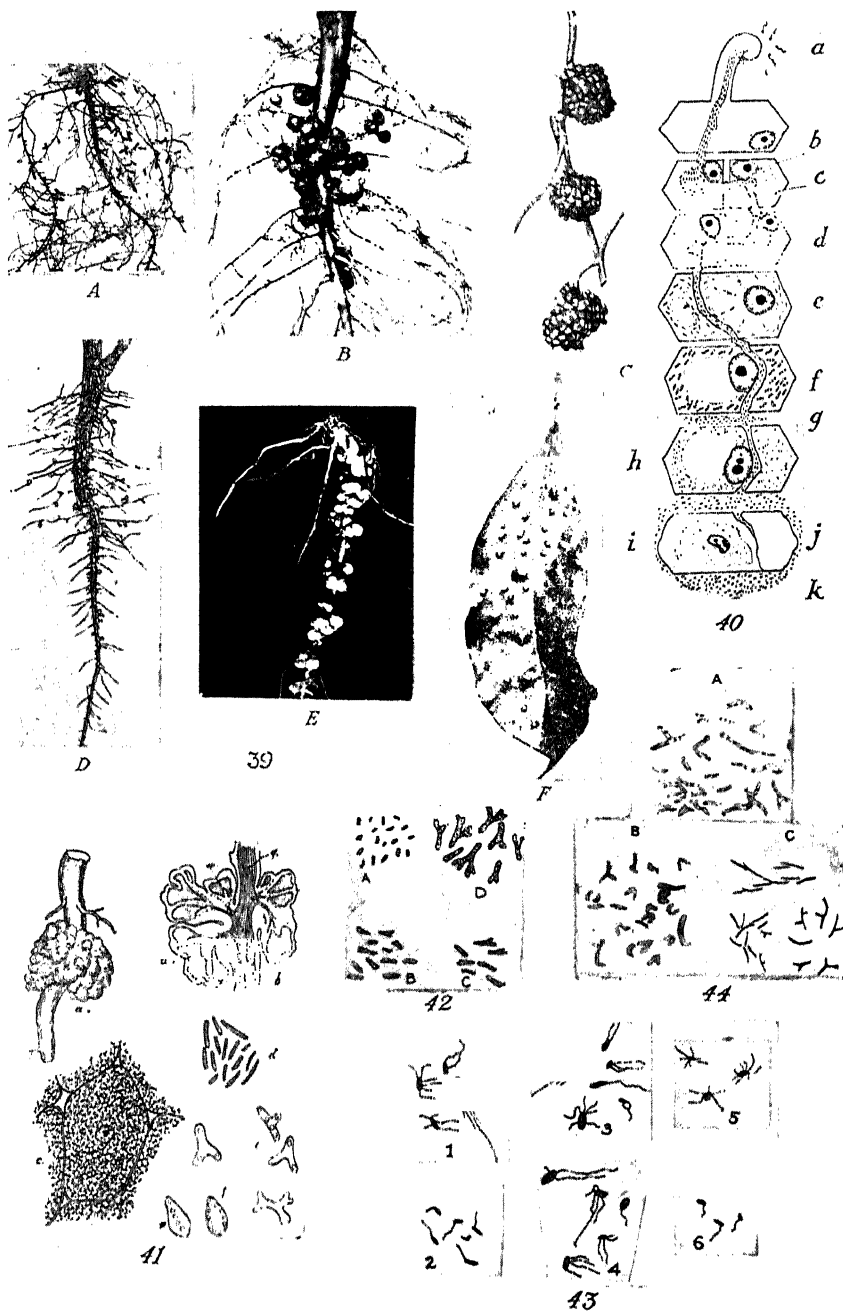
40. Life history of *Bact. radiculicola* in the tissue of the lucerne nodule (from Thornton): a. infected root hair; b. host cell nucleus; c. infection thread; d. release of coccoid rods from infection thread; e. multiplication of bacteria in the cytoplasm; f. formation of swollen banded rods in the cytoplasm; g. invasion of cell walls from infected thread; h. banded rods breaking up into granules; i. disappearance of granules; j. disintegration of old nodule tissue; k. release of coccoid rods into the soil.

41. Detailed examination of *Bact. radiculicola* on lupines: a. nodule, natural size; b. nodule bacteria,  $\times 2100$ ; e-f, bacteroids,  $\times 1200$  (after Woronin, Fischer and Omeliansky).

42. Nodule bacteria of *Vicia sativa*: a-d, transformation of rods into bacteroids (after Beijerinck).

43. Flagellation of bacteria of leguminosae: 1. *Phaseolus vulgaris*; 2. *Cracca virginiana*; 3. *Vicia sativa*; 4. *Medicago sativa*; 5. *Melilotus alba*; 6. *Lespedeza striata* (from Shunk).

44. *Bact. rubiacearum* of *Pavetta zimmermanni*: A. contents of nodule; B. preparation of colony grown on agar; C. various forms of different stages of development of pure culture: A and B,  $\times 1660$ ; C,  $\times 1300$  (after von Faber and de Rossi).







showed a gain of 34.1 and 40.6 mgm. of nitrogen and abundant nodule formation. Nobbe and Hiltner<sup>92</sup> concluded that the fixation of nitrogen by leguminous plants is closely related to the formation of bacteroids in the nodules.

*Nomenclature.* The causative agent of the nodules on the roots of leguminous plants is referred to by different names, depending on the particular system of classification. In 1879, Frank<sup>93</sup> described the nodule-forming "fungus" as *Schinzia leguminosarum*. However, in 1888, Beijerinck recognized that the causative agent is a bacterium, which he named *Bacillus radicola*. In view of the fact that the organism is non-spore forming and is destroyed at 60° to 70°C., Prazmowski changed its name to *Bacterium radicola*. In 1890, Frank<sup>93</sup> considered the infecting threads to consist of a mixture of protoplasm of the host cells and of the infecting organism which was called *Rhizobium leguminosarum*. Other names were given to this organism. The fact that a number of races produce only a single polar flagellum led various investigators<sup>94</sup> to classify it with the genus *Pseudomonas*, under the name of *Pseudomonas radicola*. E. F. Smith<sup>95</sup> and the Committee of the Society of American Bacteriologists (p. 56) decided that the name given to the organism by Frank in 1879 deserves priority; *Bacterium leguminosarum* or *Rhizobium leguminosarum* was therefore recognized as the correct name.

Löhnis and Hansen were of the opinion<sup>96</sup> that the nodule bacteria do not represent a special genus *Rhizobium*, but are closely related to *Bact. radiobacter*, *Bact. lactis viscosum*, *Bact. pneumoniae* and *Bact. aerogenes*, the last three being immotile and the first motile. The species differ only to a slight extent in their physiological and morphological characters. These closely related forms are well distributed in the soil and *Bact. radiobacter* may actually be present in the root nodules of leguminous plants. On account of its resemblance to *Bact. radicola*, it has been mistaken for the nodule-producing organism in the cowpea-soybean group, since it grows rapidly on the plates made from the nodules; however, it can be differentiated from the latter by its growth on milk.

<sup>92</sup> Nobbe, F. and Hiltner, L. Landw. Vers. Sta. **42**: 459-478. 1893.

<sup>93</sup> Frank, B. Bot. Ztg. **37**: 377-388, 393-400. 1879; Ber. deut. bot. Gesell. **7**: 234-247. 1889; Landw. Jahrb. **19**: 523-640. 1890.

<sup>94</sup> Moore, G. T. Yearb. U. S. Dept. Agr. for 1902, 333-342; Burrill, T. J. and Hansen, R. Ill. Agr. Exp. Sta. Bul. **202**: 115-181. 1917.

<sup>95</sup> Smith, E. F. Bacteria in relation to plant diseases. Washington, **2**: 97-146. 1921.

<sup>96</sup> Löhnis, F. and Hansen, R. Jour. Agr. Res. **20**: 543-556. 1921.

*Media.* A number of media have been suggested, at various times, for the cultivation of the organism causing the nodules on leguminous plants. In addition to various organic media, extracts of carrots, of leaves and of seeds of leguminous plants, a number of inorganic media have been suggested. Of these, several may be selected:

1. Wood ash medium:<sup>97</sup>

Wood ash extract (15 grams ashes to 1 liter tap water).....	1000 cc.
Sucrose.....	10 grams
KH <sub>2</sub> PO <sub>4</sub> .....	3 grams

2. Ashby's mannitol solution (p. 109).

3. Conn's asparaginate solution (p. 16).

4. Glucose medium:<sup>98</sup>

Distilled water.....	1000 cc.	NaCl.....	Trace
Glucose.....	20 grams	FeSO <sub>4</sub> .....	Trace
KH <sub>2</sub> PO <sub>4</sub> .....	1.0 gram	MnSO <sub>4</sub> .....	Trace
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.1 gram	CaCl <sub>2</sub> .....	Trace

5. Sucrose medium:

Tap water.....	1000 cc.	KH <sub>2</sub> PO <sub>4</sub> .....	1.0 gram
Sucrose.....	10 grams	MgSO <sub>4</sub> .....	0.5 gram

6. Mannitol medium:<sup>99</sup>

Mannitol.....	10 grams	CaCO <sub>3</sub> .....	1.0 gram
NaCl.....	0.2 gram	Yeast water.....	100 cc.
K <sub>2</sub> HPO <sub>4</sub> .....	0.5 gram	Distilled water.....	900 cc.
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.2 gram	Washed agar.....	15 grams
CaSO <sub>4</sub> ·2H <sub>2</sub> O.....	0.1 gram		

The yeast water is prepared<sup>100</sup> by stirring starch-free yeast with ten times its weight of tap water, steaming for 1 to 2 hours, then sterilizing and, after allowing to stand 24 hours, siphoning off the clear brown liquid.

Various legume extract and tomato extract media are also employed: A decoction of 100 grams material of the green plants and roots in 1000 cc. of water, to which 1 per cent glucose is added and some CaCO<sub>3</sub> to make the reaction neutral.<sup>101</sup> For solid media, 1.2 to 1.5 per cent of agar is used; for gelatin media, 12 per cent of gelatin is used. When the reaction is adjusted by the hydrogen-ion concentration method, it should be brought to pH 6.8 to 7.5.

*Nodule formation.* The bacteria usually enter the plant through the root hairs or through other epidermal cells. On entering the root, the bacteria multiply, forming a thread of infection, which branches out into

<sup>97</sup> Harrison, F. C. and Barlow, B. *Centrbl. Bakt.* II, 19: 264-272, 426-441. 1907; *Trans. Roy. Soc. Can. Ser. (2)*, 12: 157-237. 1907.

<sup>98</sup> Fred, E. B. *Va. Agr. Exp. Sta. Ann. Rpt.* 1911, 145-174.

<sup>99</sup> Wright, W. H. *Soil Sci.* 20: 95-120. 1925.

<sup>100</sup> Fred, E. B., Peterson, W. H. and Davenport, A. *Jour. Biol. Chem.* 42: 175-189. 1920.

<sup>101</sup> Nobbe, F. and Hiltner, L. *Centrbl. Bakt.* II, 6: 449-457. 1900.

the parenchymatous cells of the root (No. 40, Pl. V). On reaching the inner cells of the root cortex, outside of the endodermis, the bacteria favor the multiplication of the surrounding cells which lead to the formation of a young nodule; this gives rise to a swelling on the side of the root by pushing out the overlying cortical parenchyma and epidermis.<sup>102</sup> In some plants, as *Lupinus* and *Phaseolus*, bacterial infection results in a rapid division of the infested cells thus giving rise to bacteroid tissue; in these plants the nodules usually arise in the cambium layers.<sup>103</sup> A third type of infection is known, characteristic of *Serradella*, in which the intercellular zoogloea plays the important part in the infection. The bacterial cells which enter the root in the form of swarmers change into rods and multiply rapidly in the slime filament or in the zoogloea mass; many of these rods change into branched banded rods or "bacteroids" in the nodule.

The size, form and position of nodules vary with the nature of the plant, soil in which it is grown and virulence of the bacteria.<sup>104</sup> According to Bryan,<sup>105</sup> nodule formation is greatly influenced by the reaction of the soil; alfalfa and clover produce maximum growth and number of nodules at pH 7.8, alsike and red clover at pH 5.6; the critical pH values for nodule formation are 4.0 and 9.0 to 10.0. Nodules will be formed at all temperatures at which the plant can make a growth that is at all vigorous.<sup>106</sup>

A small amount of nitrogen in soil favors nodule development, due probably to the stimulation of root growth. Large amounts of available nitrogen in the soil, such as nitrates, will retard and may even prevent, if present in sufficient quantities, nodule formation by the plant as well as nitrogen-fixation.<sup>107</sup> The injurious influence of nitrate upon nodule formation is due largely to the fact that it offers a source of available

<sup>102</sup> Brenchley, W. E. and Thornton, H. G. Proc. Roy. Soc. London B, 99: 427. 1926; Thornton, H. G. Ibid. 106: 110-122. 1930; Ann. Bot. 44: 385-392. 1930; McCoy, E. F. Centrbl. Bakt. II, 79: 394-412. 1929.

<sup>103</sup> Milovidov, P. E. Centrbl. Bakt. II, 68: 333-345. 1926. Rev. Gen. Bot. 40: 193-205. 1928.

<sup>104</sup> Hiltner, L. Lafar's Handb. techn. Mykol. 3: 24-70. 1904.

<sup>105</sup> Bryan, O. C. Soil Sci. 15: 23-35. 1923.

<sup>106</sup> Jones, F. R., and Tisdale, W. B. Jour. Agr. Res. 22: 17-31. 1921.

<sup>107</sup> Giobel, G. N. J. Agr. Exp. Sta. Bul. 436, 1926; Nobbe, F. and Richter, L. Landw. Vers. Sta. 56: 441-448. 1902; 59: 167-174. 1904; Prucha, M. J. Cornell Univ. Agr. Exp. Sta. Mem. 5, 1915; Strowd, W. H. H. Soil Sci., 10: 343-356. 1920; Wilson, J. K. N. Y. (Cornell) Agr. Exp. Sta. Res. Bul. 386. 1917; Weber, E. Centrbl. Bakt. II, 82: 353-379. 1930.

nitrogen to the plant; it may also have a specific action upon the plant juice. But other nitrogen compounds, such as ammonium salts or ammonia-producing substances, may also reduce or even inhibit nodule formation. This is probably due to the direct assimilation of the combined nitrogen by the plants rather than to any inhibition of bacteria from penetrating into the roots. Carbonaceous substances, such as carbohydrates, certain organic acids and alkaloids stimulate nodule production in the soil. However, appreciable amounts of nitrogen will be fixed even in the presence of considerable quantities of available nitrogen, including nitrates, in the soil.<sup>108</sup>

The occurrence of bacteria in the nodules of some plants, as in the case of peas, has been correlated with the ineffectiveness of such nodules; effective nodules are either filled with bacteroids, such as those usually described, or with the so-called brown bacteroids, which grow very slowly.<sup>109</sup> Certain strains of nodule bacteria are inactive, although they may produce a large number of nodules.

*Isolation of bacteria from nodules.* Harrison and Barlow described in detail the method of isolation and cultivation of the organism.

A medium sized nodule, appearing young and sound, is selected. It is cut off so as to leave 2 to 3 mm. of the root on both sides to permit handling it with forceps. The nodule is then washed, rinsed in distilled water and dropped into a sterilizing liquid containing 1 gram  $\text{HgCl}_2$  and 2.5 cc. c. p.  $\text{HCl}$  in 500 cc. of water. The nodule is well shaken in the solution for 3 to 4 minutes, then washed three times in sterile distilled water. It is then covered with about 1 cc. of sterile distilled water and crushed with a sterile, heavy glass rod. Two or three drops of the cloudy suspension are placed into a test tube of the agar medium, which has previously been liquefied and cooled to  $45^\circ\text{C}$ . A second tube of agar is then inoculated with five drops from the first; a third tube is inoculated from the second and a fourth tube from the third; the plates are poured and incubated at  $20^\circ$  to  $25^\circ\text{C}$ . The organism is isolated upon sterile agar slants or liquid media from a typical colony upon the plate, using the third and fourth plates and discarding the first two. The lens-shaped and pin-head colonies should be selected rather than the giant colonies. In case of questionable plates, replating may be necessary from the culture isolated. To keep the cultures in stock, one of the above agar media (ash or mannitol agar) may be used.

*Isolation from soil.* *Bact. radiculicola* can readily spread through the soil and persist there for a long period of time, the rate of spreading being very slow, not more than 1 inch in 24 hours.<sup>110</sup> It can be isolated

<sup>108</sup> Albrecht, W. A. Soil Sci., 9: 275-319. 1920.

<sup>109</sup> Löhnis, M. P. Centrbl. Bakt. II, 80: 342-368. 1930.

<sup>110</sup> Ball, O. M. Centrbl. Bakt. II, 23: 47-59. 1909; Kellerman, K. F. and Fawcett, E. H. Science, 25: 806. 1907; Thornton, H. G. and Gangulee, N.

from the soil and cultivated, although the specificity of the forms isolated by Nobbe and Hiltner and Gage<sup>111</sup> has not been sufficiently demonstrated. The abundance of each strain of *Bact. radicola* in soil depends upon the reaction of the soil, season of year and type of soil. An acidity greater than pH 5.4 is detrimental to the development of most strains; at a favorable reaction (pH 5.4-6.8), as many as 100,000 to 1,000,000 cells of different strains may be found per gram of soil.<sup>112</sup> The numbers are lowest in winter, and highest in June and in October. Kellerman and Leonard<sup>113</sup> could isolate the organism only from soils previously sterilized and inoculated. Nodule bacteria were isolated from soil by Beijerinck, Nobbe and others. Lipman and Fowler<sup>114</sup> employed two media for this purpose: (1) 1000 grams of water, 10 grams maltose, 1 gram  $K_2HPO_4$ , 1 gram  $MgSO_4$ , 2 to 3 drops each of 10 per cent solution of NaCl,  $FeCl_3$ ,  $MnSO_4$ , and  $CaCl_2$  and 15 grams of agar. (2) Soil extract, obtained by boiling one part of soil with three parts of water for one hour, then filtering and adding 15 grams of agar and 10 grams of maltose to 1 liter of the extract. A soil in which *Vicia sicula* has been grown a year before was used for plating out on these media. The capacity of the colonies developing on the plate to inoculate plants obtained from disinfected seed grown in sterile soil was then tested, and it was found that nearly half of the colonies were those of the organism in question.

*Colony appearance.* The colonies appearing on the plate are either surface or deep colonies. The first are drop-like, watery, mucilaginous in appearance, gray-white to pearly white in color, glistening, and semi-translucent to opaque. The edges are smooth and even; they frequently attain a size of 1 cm. or more in diameter. The deep colonies are small, lens or spindle shaped, with smooth and even edges, opaque, granular in structure, and cream colored to chalky white. They slowly increase in size, eventually appearing on the surface, when growth becomes rapid. When first isolated, colonies may not appear before 6 to 14 days. Differ-

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Proc. Roy. Soc. London B, 99: 427. 1926; see also Frazier, W. C. and Fred, E. B. Soil Sci. 14: 29-35. 1922.

<sup>111</sup> Nobbe, Schmid, Hiltner and Hotter. Landw. Vers. Sta. 39: 327-359. 1891. Gage, G. E. Centrbl. Bakt. II, 27: 7-48. 1910.

<sup>112</sup> Wilson, J. K. Jour. Amer. Soc. Agron. 18: 911-919. 1926; Soil Sci. 30: 289-296. 1930; Wilson, P. W. and Kullmann, E. D. Jour. Bact. 22: 71-90. 1931.

<sup>113</sup> Kellerman, K. F. and Leonard, L. T. Science, N.S. 38: 95-98. 1913.

<sup>114</sup> Lipman, C. B. and Fowler, L. W. Science, N. S. 41: 256-259, 725. 1915; see Allen, O. N. and Baldwin, I. L. Jour. Amer. Soc. Agron. 23: 28-31. 1931.

ent strains vary markedly in this respect. The colonies from alfalfa, clover and pea nodules are large, opaque, sticky; the colonies from soybeans are small and produce a transparent film slowly spreading over the surface of the agar medium. The first group contains organisms which grow much faster than others. To the slow growers belong, in addition to the soybean, also the cowpea and others (No. 49, Pl. VI).

*Morphology and life cycle of nodule bacteria.* The organism varies greatly in size and shape in the nodule. Many small, oval forms, described by Beijerinck as swimmers and normal rods, are found together with a few large club-shaped or branching forms (bacteroids) in the young nodules. In the old, decomposing nodule, the branching forms are extremely vacuolated, showing small, oval, deep staining bodies within.<sup>115</sup> These bodies may be the motile swimmers or the branching form dividing into bacilli.

In pure cultures, the organism forms minute short rods, motile when young by means of flagella.<sup>116</sup> The bacteroids may be produced also on artificial culture media in the presence of acid phosphate, sodium succinate and glycerol, caffeine and cumarine.<sup>117</sup> According to Barthel,<sup>118</sup> caffeine and other vegetable alkaloids, like guanidine, pyridine and chinoline, will stimulate the formation of involution forms in pure culture; he suggested, therefore, that the formation of these so-called bacteroids in root nodules is due to the presence of alkaloids in the plant. The bacteroids are never so large and numerous on the artificial culture media as in a young nodule; they are produced, either in the medium, or in the nodule due to specific nutrition or to unfavorable conditions. According to Zipfel, the branching forms are not degeneration forms, but may be looked upon as a normal and necessary stage in the life of the organism with specific biological functions; they are formed from rods and change again into rods when inoculated into proper media.

Five stages in the life cycle of the *Bact. radiculicola*, through which it passes under cultural conditions, were recognized.<sup>119</sup>

<sup>115</sup> de Rossi, G. Centrbl. Bakt. II, 18: 289-314, 481-489. 1907.

<sup>116</sup> Barthel, Chr. Ztschr. Gärungsphys. 6: 13. 1917.

<sup>117</sup> Stutzer, A. Centrbl. Bakt. II, 7: 897-912. 1901; Buchanan, R. E. Centrbl. Bakt. II, 23: 59-91. 1909; Zipfel, H. Centrbl. Bakt. II, 32: 97-137. 1912; Fred, 1911 (p. 122).

<sup>118</sup> Barthel, C. Ann. Inst. Past. 35: 634-647. 1921.

<sup>119</sup> Bewley, W. F. and Hutchinson, H. B. Jour. Agr. Sci. 10: 144-162. 1920; Thornton and Gangulee, 1926 (p. 124); Gibson, T. Jour. Agr. Sci. 18: 76-88. 1928.

1. Non-motile, pre-swarmer form, obtained in 4 to 5 days when a culture of the organism is placed in a neutral soil solution.

2. Larger, non-motile coccus. The pre-swarmer coccoid changes in the presence of saccharose, certain other carbohydrates and phosphates, by increasing in size until the diameter has doubled.

3. Motile, swarmer stage, when the cell becomes ellipsoidal and develops high motility.

4. Rod-form, as a result of the further elongation of the swarmer, with decreasing motility.

5. Vacuolated stage. When available carbohydrates become exhausted or the organism is placed in a neutral soil extract, the cell becomes highly vacuolated and the chromatin divides into a number of bands. Finally these bands become rounded off and escape from the rod as the coccoid pre-swarmer. The pre-swarmer stage is usually formed from normal rods in calcareous soils, when calcium or magnesium carbonates are added to the medium, or under anaerobic conditions. Acid soils cause the production of highly vacuolated cells and eventually kill the organism.

*Motility.* In young agar slants, *Bact. radicola* is found to be very motile. Owing to the slime produced by the organism, the demonstration of flagella is very difficult; this was the reason for considerable disagreement among the different investigators. It has come to be recognized,<sup>120</sup> however, that the nodule bacteria possess two types of flagellation: peritrichous and monotrichous. The alfalfa, clover, pea and bean groups possess numerous peritrichous flagella; the cowpea and soybean groups are monotrichous.<sup>121</sup> Differences, however, have been reported even for a single strain; the soybean organism may also possess peritrichous flagellation.<sup>122</sup> The difference thus obtained may be due either to the fact that cultures of various ages were employed or that different types of bacteria exist, even for the same plant in different parts of the world.<sup>123</sup>

For staining of flagella, the following modification of the Loeffler's stain may be used:

*Solution A*

	<i>parts</i>
Ferric chloride (1:20 aqueous solution).....	1
Saturated aqueous solution of tannic acid.....	3

This solution improves with age; it should be at least a week or two old and should be filtered before using.

<sup>120</sup> Hansen, R. Science, N. S. 50: 568-569. 1919.

<sup>121</sup> Wright, 1925 (p. 122).

<sup>122</sup> Wilson, J. K. Cornell Univ. Exp. Sta. Bul. 386. 1917.

<sup>123</sup> Shunk, I. V. Jour. Bact. 6: 239-246. 1921; Ibid. 5: 181-187. 1920; Fred and Davenport, 1918 (p. 128).



*Solution B*

	<i>parts</i>
Aniline oil.....	1
95 per cent alcohol.....	4

The bacterial suspension is allowed to air-dry on a clean cover glass. About 5 drops of solution A are then placed on the cover glass, followed immediately by 1 to 2 drops of solution B. The combination is allowed to act at room temperature for 2 minutes and is then washed in distilled water. The stain (30 parts of saturated alcoholic solution of methylene blue, 13 parts of solution B as mordant and 100 parts of 1:10,000 KOH solution) is applied for 2 minutes.

Löhnis and Hansen and Shunk observed the two distinct types of flagellation referred to above. In the single flagellate types (monotrichous), the flagellum is not strictly polar but is usually attached to the corner (No. 43, Pl. V).

*Physiology of nodule bacteria.* The different strains of *Bact. radiculicola* are strictly aerobic. Maltose, sucrose, glucose and mannitol offer the best sources of carbon; lactose, dextrin and glycerol can also be utilized. According to Beijerinck, separate carbon and nitrogen sources (asparagine, ammonium sulfate, sodium or potassium nitrate) are required. Laurent<sup>124</sup> first showed that the organism can be cultivated on nitrogen-free media, containing 0.1 per cent  $\text{KH}_2\text{PO}_4$ , 0.01 per cent  $\text{MgSO}_4$  and 5 to 10 per cent of an available energy source. Some of the bacteria produce considerable acidity while others do not. This corresponds with the flagellation of the strains, the acid producers being peritrichous.<sup>125</sup> *Bact. radiobacter* can be readily distinguished from the root nodule bacteria by its strong acid production from dextrin.

The optimum reaction for the growth of the bacteria is pH 5.5 to 7.0, depending on the nature of the plant, with limiting reactions of pH 3.2 to 5.0 on the acid side, and pH 9.0 to 10.0 on the alkaline. Mazé<sup>126</sup> was the first to call attention to the fact that the nodule bacteria comprise both acid resistant and acid sensitive types. The alfalfa organism is most sensitive to acidity and the lupine organism is most resistant, as seen from the following summary:<sup>127</sup>

<sup>124</sup> Laurent, E. Compt. Rend. Acad. Sci. 111: 754. 1890; Ann. Inst. Past. 4: 722-744. 1890; 5: 105-139. 1891.

<sup>125</sup> Baldwin, I. L. and Fred, E. B. Soil Sci. 24: 217-230. 1927.

<sup>126</sup> Mazé, P. Ann. Inst. Past. 13: 145-155. 1899.

<sup>127</sup> Fred, E. B. and Davenport, A. Jour. Agr. Res. 14: 317-336. 1918; Bryan, O. C. Soil Sci. 15: 37-40. 1923.

	<i>Limiting pH</i>
1. Alfalfa and sweet clover organism.....	4.9
2. Garden pea, field pea and vetch.....	4.7
3. Red clover and common beans.....	4.2
4. Soybeans and velvet beans.....	3.3
5. Lupines.....	3.15

Different biological types of alfalfa and soybean organisms may vary in their limiting reaction.<sup>128</sup> One strain of the soybean organism was found to have its acid limit at pH 4.0 to 4.5 and the other at pH 4.5 to 5.0. The optimum reaction depends also on the strain.

The limiting temperatures for the growth of nodule bacteria are 3° and 46°C.; the thermal death point is at 60° to 62° while the optimum varies between 18° and 26°C.<sup>129</sup> The bacteria are not injured by diffused sunlight and can withstand readily direct sunlight. Drying is injurious<sup>130</sup> but not fully destructive.<sup>131</sup> As a result of direct and rapid drying of soil, the numbers of *Bact. radiculicola* diminish rapidly, as determined by the plate method; however, a much greater number actually remain alive but unable to develop on the plate into colonies.<sup>132</sup> The organism will persist in soil for at least several years, even in the absence of the host plant.

The nodule bacteria can be modified in their ability to grow under unfavorable conditions; a character, such as tolerance to dyes, may be modified relatively quickly.<sup>133</sup> The character which has been lost as a result of cultivation on artificial media is quickly regained when the culture is returned to the soil.

*Specific differentiation.* Three groups of methods are usually employed for the specific differentiation of the nodule bacteria: (1) plant inoculation, (2) morphological and cultural studies, (3) serological and immunological reactions. Although Nobbe, Hiltner and Schmid<sup>134</sup> came to the conclusion that the bacteria in the nodules of all legumes are strains of the same organism, the fact was soon brought to light that not all the bacteria obtained from the nodules of various plants can cross-inoculate and produce nodules on the roots of other leguminous

<sup>128</sup> Stevens, J. W. *Soil Sci.* **20**: 45-66. 1925; Wright, W. H. **20**: 95-120. 1925.

<sup>129</sup> Zipfel, 1912 (p. 126); Vogel, J. and Zipfel, H. *Centrbl. Bakt.* **II**, **54**: 13-34. 1921.

<sup>130</sup> Chester, F. B. *Del. Agr. Exp. Sta. Bul.* **78**. 1907.

<sup>131</sup> Ball, O. M. *Centrbl. Bakt.* **II**, **23**: 47-59. 1909.

<sup>132</sup> Duggar, B. M. and Prucha, M. J. *Centrbl. Bakt.* **II**, **34**: 67. 1912.

<sup>133</sup> Burke, V. and Burke, L. *Soil Sci.* **20**: 143-146. 1925.

<sup>134</sup> Nobbe, F., Hiltner, L. and Schmid, E. *Landw. Vers. Sta.* **45**: 1-27. 1895.

plants. These plants could readily be divided into several closely related groups, those belonging to each group having their own specific organism; cross inoculation can take place only by the members of each group.

Frank was the first to recognize, in 1879, that a difference exists between the type of nodules produced on *Lupinus* and *Lathyrus*, as well as between the organisms present in these nodules. Schroeter<sup>135</sup> suggested the separation of the bacteria into two species. *Phytomyxa leguminosarum* (Frank) Schr. for the form causing nodules on *Trifolium repens*, *Orobis vernus*, etc., and *Phytomyxa lupini* Schr. causing nodules on *Lupinus luteus* and *L. angustifolius*. Beijerinck recognized in 1888 that not all the strains of the nodule organism are identical; he listed seven varieties of *Bacillus radiculicola*. Schneider<sup>136</sup> described in 1892 five species of nodule bacteria, with two varieties of one. Hiltner and Störmer<sup>137</sup> distinguished, on the basis of morphological and cultural studies, two groups of bacteria: (1) *Bact. radiculicola* on *Pisum*, *Vicia*, *Lathyrus*, *Phaseolus*, *Trifolium*, etc., and (2) *Bact. beijerinckii* on *Lupinus*, *Ornithopus*, *Glycine*. The former grows well on certain gelatin media and readily produces branching forms, while the latter grows poorly on gelatin media. It was soon found that a further subdivision would have to be made, *Pisum*, *Trifolium*, *Medicago* and *Lupinus* bacteria being taken as representative types.

Zipfel<sup>129</sup> made use of agglutination tests and concluded that nodule bacteria were not varieties of the same species, but that distinct species existed. Six groups were thus recognized: (1) *Lupinus*, (2) *Trifolium*, (3) *Medicago*, (4) *Pisum*, (5) *Faba*, and (6) *Phaseolus*. On the basis of serological investigation, Klimmer and Krüger<sup>138</sup> formed nine groups of legume bacteria: (1) *Lupinus* and *Ornithopus*, (2) *Melilotus*, *Medicago*, and *Trigonella*, (3) *Vicia* (*V. sativa*), (4) *Pisum*, (5) *Vicia faba*, (6) *Trifolium pratense*, (7) *Phaseolus vulgaris*, (8) *Soja hispida*, and (9) *Onobrychis sativa*. Other serological studies<sup>139</sup> confirmed the general conclusion that the nodule bacteria include more than one organism.

The agar test-tube method may be used for the study of nodule forma-

<sup>135</sup> Schroeter, J. Cohn's Krypt. Flora von Schlesien. 9: 135. 1886.

<sup>136</sup> Schneider, A. Bull. Tor. Bot. Club. 19: 203-218. 1892.

<sup>137</sup> Hiltner, L. and Störmer, K. Arb. k. Gesundheitsamt., Biol. Abt. 3: 151-307. 1903.

<sup>138</sup> Klimmer, M. and Krüger, R. Centrbl. Bakt. II, 40: 256-265. 1914; Klimmer, M. Centrbl. Bakt. II, 55: 281-283. 1922; Simon, J. Centrbl. Bakt. II, 41: 470-479. 1914.

<sup>139</sup> Stevens, J. W. Jour. Inf. Dis. 33: 557-566. 1923; Soil Sci. 20: 45-66. 1925.

tion on the roots of certain legumes by different strains of bacteria.<sup>140</sup> On the basis of the cultural method, the nodule bacteria were divided into the following groups: (1) alfalfa organism inoculating also *Medicago lupulina*, *M. denticulata* and *Melilotus*, (2) clover organism inoculating all species of *Trifolium*, (3) vetch and garden pea, (4) cowpea, (5) soybean, (6) garden bean. Burrill and Hansen<sup>141</sup> demonstrated, by cross-inoculation studies, eleven kinds of bacteria divided into three groups, namely: (1) thin, scant, slow growth on ash-agar slant; little gum formed, flagella easily demonstrated—*Vigna*, *Cassia*, *Acacia*, *Glycine*, etc.; (2) more rapid and more abundant growth; glistening, opaque and pearly white; considerable gum formed which interferes with attempt of staining flagella—*Melilotus*, *Medicago*, *Trigonella*; (3) very fast, spreading growth; watery and semi-translucent; very slimy and sticky, due to excess of gum—*Vicia*, *Pisum*, *Lens*, *Lathyrus*, *Trifolium*, *Phaseolus* and *Strophostyles*.

Löhnis and Hansen<sup>142</sup> divided the bacteria of the leguminous plants into two groups, the representatives of which differ both morphologically and physiologically. The first group shows all the features of *Bact. radiculicola*; it is peritrichic, grows relatively fast on agar plates and changes milk characteristically; it produces nodules on the roots of clover, sweet clover, alfalfa, vetch, pea, navy bean, lupine, black locust, *Amorpha* and *Strophostyles*. The second group is characterized by monotrichic flagellation, comparatively slow growth on agar plates, and inability to cause a marked change in milk; it has been isolated from the soybean, cowpea, lima bean, peanut, beggarweed, *Acacia*, *Genista*, *Cassia* and *Amphicarpa*. However, they did not suggest separating the organism into two new species before the complete life history of the two groups is known.

Bergey<sup>143</sup> placed the *Bact. radiculicola* in a separate genus *Rhizobium*, and separated the different forms into two species: (1) *Rh. leguminosarum* Frank, inoculating *Pisum*, *Vicia*, *Lathyrus*, etc., (2) *Rh. radiculicola* Beij. of *Trifolium*, *Phaseolus*, etc.

The following is a list of leguminous plants, divided on the basis of inter-inoculation.<sup>144</sup> The different members in any one group are those which can be inoculated by the strain of the *Bact. radiculicola* specific for that group.

<sup>140</sup> Garman, H. and Didlake, M. Ky. Agr. Exp. Sta., Bul. 184: 343-363. 1914.

<sup>141</sup> Burrill and Hansen, 1917 (p. 121).

<sup>142</sup> Löhnis and Hansen, 1921 (p. 114).

<sup>143</sup> Bergey, 1930 (p. xi).

<sup>144</sup> Hansen, R. Sci. Agr. (Canada) 1: 59-62. 1921; Whiting, A. L., Fred, E. B. and Helz, G. E. Soil Sci. 22: 467-476. 1926.

## Group I:

*Trifolium pratense*, red clover  
*Trifolium hybridum*, alsike clover  
*Trifolium alexandrinum*, berseem clover  
*Trifolium incarnatum*, crimson clover  
*Trifolium repens*, white clover  
*Trifolium medium*, zigzag, or cow clover

## Group II:

*Melilotus alba*, white sweet clover  
*Melilotus officinalis*, yellow sweet clover  
*Medicago sativa*, alfalfa  
*Medicago hispida*, bur clover  
*Medicago lupulina*, black medick, or yellow trefoil  
*Trigonella foenum-graecum*, fenu-greek

## Group III:

*Vigna sinensis*, cowpea  
*Cassia chamaecrista*, partridge pea  
*Arachis hypogaea*, peanut  
*Lespedeza striata*, japan clover  
*Mucuna utilis*, velvet bean  
*Baptisia tinctoria*, wild indigo  
*Desmodium canescens*, tick trefoil  
*Acacia armata*, acacia  
*Genista tinctoria*, dyer's greenwood  
*Phaseolus lunatus*, lima bean

## Group IV:

*Pisum sativum arvense*, Canada field pea  
*Vicia villosa*, hairy vetch  
*Vicia sativa*, spring vetch  
*Vicia faba*, broad bean  
*Lens esculenta*, lentil  
*Lathyrus latifolius*, sweet pea

## Group V:

*Glycine hispida* (*Soja max*), soybean

## Group VI:

*Phaseolus vulgaris*, garden bean  
*Phaseolus multiflorus*, scarlet runner bean

## Group VII:

*Lupinus perennis*, lupine  
*Ornithopus sativa*, serradella

## Group VIII:

*Amphicarpa monoica*, hog peanut

## Group IX:

*Amorpha canescens*, lead plant

## Group X:

*Strophostyles helvola*, trailing wild bean

## Group XI:

*Robinia pseudo-acacia*, black or common locust

## Group XII:

*Dalea alopecuroides*, wood's clover

A similar list was prepared by Müller and Stapp,<sup>145</sup> while Dangeard<sup>146</sup> actually decided that at least 10 species should be recognized. Baldwin and Fred<sup>147</sup> recognized for the present only five species, namely,

*Rhizobium leguminosarum* Frank causing the formation of nodules upon the roots of the plants included in the above group IV.

*Rhizobium trifolii* Dangeard (on plants of Group I).

*Rhizobium phaseoli* Dangeard (on plants of Group VI).

*Rhizobium meliloti* Dangeard (on plants of Group II).

*Rhizobium japonicum* (Kirchner) on the roots of *Soja max*.

<sup>145</sup> See Müller, A. and Stapp, C. Arb. Biol. Reichsanst. Land-Forstw. 14: 455-554. 1925.

<sup>146</sup> Dangeard, M. P. A. Le Botaniste, Ser. 16: 1-270. 1926.

<sup>147</sup> Baldwin, I. L. and Fred, E. B. Jour. Bact. 17: 141-150. 1929.

It has been claimed, as a result of laboratory studies, that the soybean and cowpea organisms show an interchangeability;<sup>148</sup> however, field tests from other sources do not indicate such relationship.<sup>149</sup>

Various explanations have been suggested for the specificity of the various root nodule organisms, based on soil reaction, climate, etc. Burrill and Hansen properly believed that it may be a case of specific enzymes produced by the bacteria or of differences in the root-sap, which cannot be detected by chemical methods. It has been recently shown<sup>150</sup> that all members of each cross-inoculation group are closely related with respect to the protein characteristics of their seeds.

The application of serological reactions has brought out the fact that various strains of bacteria may form nodules on the same host plant but only one serological type is found in the same nodule.<sup>151</sup> The fact that not all strains of *Bact. radicicola* of one leguminous plant are identical suggested the existence of various biotypes even for the same plant. Two general types of the organism which can form nodules on the soy bean, identical morphologically but different physiologically and especially serologically, have been demonstrated.<sup>152</sup>

These results are probably due to the fact that a bacterial culture is actually a population in which the different cells have variable properties. Although morphology may not be sufficient to demonstrate any differences between the members of the population, physiological reactions and the even more sensitive serological reactions can bring out these variations. This explains the modification of a strain when grown on artificial culture media or as a result of repeated passage through the host plant. It also suggests the possibility of improving or deteriorating a strain by the proper selection of the types of cell. This phenomenon explains the increase in activity and fixation of nitrogen by repeated passage through plants.<sup>153</sup> The process of adaptation to a particular host plant is longer in case of vegetatively weak organisms than for vegetatively strong organisms.

A detailed study of the chemistry of nitrogen fixation by nodule bacteria and the artificial inoculation of soil with bacterial cultures will be discussed elsewhere.

<sup>148</sup> Leonard, L. T. Soil Sci. 15: 277-283. 1923.

<sup>149</sup> See Sears, O. H. and Carroll, W. R. Soil Sci. 24: 413-420. 1927.

<sup>150</sup> Baldwin, I. L., Fred, E. B. and Hastings, E. G. Bot. Gaz. 83: 217-243. 1927.

<sup>151</sup> Bialosuknia, W. and Klott, C. Roczn. Nauk. Rolniczych. 9: 288-335. 1923.

<sup>152</sup> Stevens, 1923-25 (p. 130); Wright, 1925 (p. 122).

<sup>153</sup> Wunschik, H. Centrbl. Bakt. II, 64: 395-445. 1925; Allen, O. N. and Baldwin, I. L. Res. Bul. 106, Wis. Agr. Exp. Sta. 1931.

*Nodule formation by non-leguminous plants.* In addition to the legumes, a number of non-legumes are found possessing nodules on their roots. Of these, most attention has been paid to *Ceanothus* (red-root), *Elaeagnus* (silver berry), *Alnus* (alder), *Coriaria*, and *Myrica* (sweet gale). The nodules produced by these plants are perennial and branch dichotomously in all directions, finally developing round aggregates of considerable size.

At first these nodules were thought to be of fungus origin. It has been found later, however,<sup>154</sup> that the nodules of *Alnus*, *Elaeagnus* and *Ceanothus* are caused by bacteria closely resembling the *Bact. radiculicola* group and capable of fixing nitrogen. However, Burrill and Hansen<sup>155</sup> stated definitely that the nodules of *Ceanothus*, *Cycas*, *Alnus* and *Myrica* are not caused by *Bact. radiculicola*. The very proof that any of these plants as well as *Elaeagnus* and *Podocarpus* are capable of fixing atmospheric nitrogen is not conclusive. In some plants (*Myrica*) the organism seems to be of the nature of an Actinomyces. *Coriaria japonica* produces nodules similar to those produced by *Alnus*, due also possibly to an Actinomyces (*Act. myricae* according to Peklo).<sup>156</sup> When possessing root nodules, this plant is able to show vigorous growth and accumu-

<sup>154</sup> Hiltner, L. Landw. Vers. Sta. **46**: 153-161. 1896; Kellerman, K. F. Yearb. Dept. Agr. U. S. A., 1910, 213-218; Bottomley, W. B. Ann. Bot. **29**: 605-610. 1915; Hocquette, M. Compt. Rend. Soc. Biol. **101**: 698-699. 1929.

<sup>155</sup> Burrill and Hansen, 1917 (p. 121).

<sup>156</sup> Arzberger, E. G. Mo. Bot. Gard. 21 Ann. Rpt.: 60-103, 1910; Shibata, K. and Tahara, M. Bot. Mag. Tokyo, **31**: 157-182. 1917.

## PLATE VI

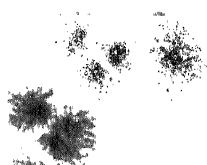
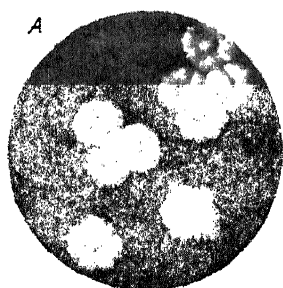
45. *Mycobacterium phlei*: A, colony of organism on washed agar containing inorganic salts, with petroleum vapor as the only source of energy; B, colony on agar with inorganic salts and 1 per cent glycerol (after Söhngen and de Rossi).

46. Life cycle of *Azotobacter chroococcum*: a, formation of symplasm by regenerative bodies on potato, in 9 days; b, regenerative units starting to grow, on beef gelatin, in 4 weeks; c, regenerative bodies growing from symplasm, on beef agar, in 4 weeks; d, formation of new cells by agglomeration of regenerative units on mannitol soil extract, in 4 days;  $\times 600$  (from Löhnis and Smith).

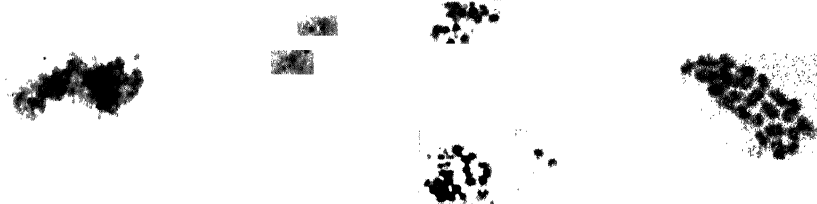
47. Deep agar colonies of anaerobic bacteria: *Bac. putrificus* in glucose agar, 48 hours old (from Weinberg and Seguin).

48. Deep agar colonies of anaerobic bacteria: *Bac. perfringens* in nitrate glucose agar (from Weinberg and Seguin).

49. Ash-agar plate showing the organism forming nodules on the roots of a, *Genista tinctoria*, 25 days old; b, *Pisum sativum*, 7 days old (from Burrill and Hansen).



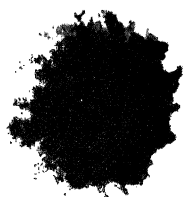
45



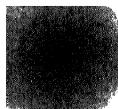
B

C

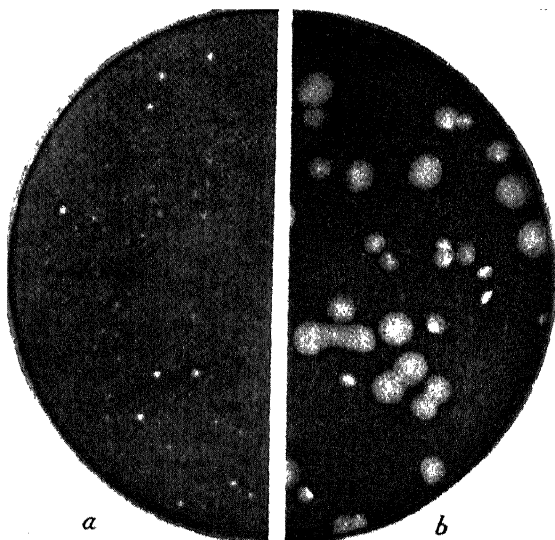
46



47



48



a

b

49





late nitrogen in a medium free from combined nitrogen, while the plants free from such nodules show all signs of nitrogen starvation.<sup>157</sup>

In the roots of *Cycas*, *Bact. radiculicola*, *Azotobacter* and algae (*Anabaena*, *Nostoc*) were demonstrated.<sup>158</sup> It is still questionable whether nitrogen fixation by most of these plants takes place,<sup>159</sup> although it is claimed that some plants (like *Casuarina*) are thus able to grow readily in very poor sandy soil.

It is of interest to point out, in this connection, that there are leguminous plants, which do not form any nodules. These include *Gymnocladus*, *Cercis* and *Gleditsia* of the subfamily *Caesalpinaceae*.<sup>160</sup>

*Nodule formation in the leaves of some plants.* A condition similar to nodule formation by bacteria on the roots of leguminous plants has been observed on the leaves of certain tropical plants, namely the *Myrsinaceae*, such as *Ardisia*, certain *Rubiaceae*, such as *Pavetta* and *Grumilea*. Koorders<sup>161</sup> demonstrated the presence of either bacteria or fungi in the bloom bud hydathodes of nineteen species of tropical plants, representing six genera; a symbiotic relation was found to exist between the host plant and the microorganisms. Zimmermann<sup>162</sup> was the first to show that the nodules on the leaves of the *Rubiaceae* (four species examined) are filled with bacteria. He also found nodules on the upper side of the leaf of *Pavetta lanceolata* and on the under side of *P. angustifolia*. The bacteria were present in chains and as longer forms. *P. indica* had even a greater number of nodules scattered over the whole surface of both sides of the leaf and formed dark green spots. The bacteria do not penetrate the cell but are found in the intra-cellular spaces. An organism belonging to the mycobacteria (*Mycob. rubiacearum*) was isolated<sup>163</sup> from the leaf nodules. The same organism was also isolated from *Pavetta* and other plants. Miehe<sup>164</sup> isolated a rod-shaped organism, *Bac. foliicola*, active in forming nodules on the leaves of *Ardisia*. It is a motile rod (1-2.5 by 0.4-0.5 $\mu$ ) with peritrichic flagella and later changes into a branching form. These "bacteroids" may be

<sup>157</sup> Kataoka, T. Japanese Jour. Bot. 5: 209-218. 1930.

<sup>158</sup> Spratt, E. R. Ann. Bot. 26: 801-814. 1912; 29: 619-626. 1915.

<sup>159</sup> Miehe, H. Flora, 111-112: 431-449. 1918; Rao, K. A. Madras Agr. Dept. Yearbook, 1923, 60-67.

<sup>160</sup> Leonard, L. T. Soil Sci. 20: 165-167. 1925; 30: 231-236. 1930.

<sup>161</sup> Koorders, S. H. Ann. Jard. Bot. Buitenzorg, 14: 354-477. 1897.

<sup>162</sup> Zimmermann, A. Jahrb. wiss. Bot. 37: 1-11. 1902.

<sup>163</sup> von Faber, F. C. Jahrb. wiss. Bot. 54: 243-264. 1914; 51: 285-295. 1912.

<sup>164</sup> Miehe, H. Jahrb. wiss. Bot. 53: 1-54. 1913; 58: 29. 1917. Ber. deut. Bot. Gesell 29: 156. 1911; 34: 576. 1916.

found in the cells of the leaves and also on special media. The bacteria are already present in the seeds, between the embryo and the endosperm, so that the plants do not have to be inoculated anew with each new growth. In this respect they are similar to the endotrophic mycorrhiza of certain Ericaceae which are considered elsewhere. When the young plants grow, the bacteria follow the growing tip to the new parts of the plant, as they develop. The bacteria are eventually found in the entire plant, where they develop in masses in the intracellular spaces. With the development of the fruit, the bacteria are enclosed in the embryo sack and remain with the seed. Miede concluded that *Bac. foliicola* fixed nitrogen; he recognized this phenomenon as one of hereditary symbiosis. The bacteria forming nodules on the leaves of *Pavetta indica* and *Chomelia asiatica* enter the stomata of the leaf, live there and fix the nitrogen from the air. The bacteria are found at all the life stages of the plant, symbiosis being developed to a much greater extent than in the Leguminosae and accompanying the whole life cycle of the plant. Plants freed from bacteria, by warming the seed for 25 minutes at 50°C., develop very slowly and suffer from lack of nitrogen. The bacteria are aerobic, rod-shaped cells.<sup>165</sup>

The presence of nitrogen-fixing bacteria in the swollen glands on the points of the leaves of *Dioscorea macroura* has also been demonstrated.<sup>166</sup>

<sup>165</sup> Rao, K. A. Agr. Jour. India, 18: 132-143. 1923.

<sup>166</sup> Orr, M. V. Notes from the Roy. Bot. Gard., Edinburgh, 14: 57-72. 1924.

## CHAPTER VI

### HETEROTROPHIC, AEROBIC BACTERIA REQUIRING COMBINED NITROGEN

*General classification.* The heterotrophic bacteria requiring combined nitrogen comprise the large numbers of organisms developing on the common plate used for counting bacteria and probably a still greater number of organisms, which develop very slowly or do not develop upon the plate at all. Morphologically they consist of spore-forming and non-spore forming rods, cocci and spirilli. Physiologically they take part in numerous soil processes, especially in the decomposition of both simple and complex organic substances in the soil including proteins, their derivatives, and other nitrogen compounds; celluloses, pentosans, and other complex and simple carbohydrates; fats and various other ingredients of natural organic matter. Morphology alone is an insufficient basis for the classification of these bacteria. Just as in the general classification, one must consider the various physiological processes in which they are concerned. The system used here is far from satisfactory, due to insufficient knowledge concerning the organisms themselves. This system is bound to change with the advance of our knowledge.

The difference between the aerobism and anaerobism of soil bacteria is largely one of degree and not of kind, as will be shown later. The anaerobic bacteria, especially the obligate forms and the cellulose-decomposing bacteria, will be treated separately because of their peculiar physiology and the special methods which are essential for their isolation, cultivation and study.

Of the two groups of aerobic rod-shaped bacteria, the non-spore formers are more numerous than the spore-formers. The latter usually become very active when fresh organic matter, rich in proteins, is added to the soil but they soon sporulate and generally remain in the soil in that condition until another favorable period arrives. The non-spore forming bacteria and cocci, living upon the colloidal film surrounding the inorganic soil particles, make up the bulk of the numbers of the soil population. Most of these organisms have not yet been described at all or only very insufficiently. Their physiological activities are

also insufficiently studied and their rôle in soil processes is little understood. Among the heterotrophic, aerobic bacteria requiring combined nitrogen there are a number of groups which are known to possess certain distinct characteristics and, therefore, require special consideration. Here belong the thermophilic bacteria, the mycobacteria, the denitrifying and urea bacteria.

*Spore-forming bacteria.* The heterotrophic, aerobic, spore-forming bacteria have been studied more completely than the non-spore formers or the anaerobic bacteria. This is due to the fact that they readily develop on the common gelatin and agar media, forming large characteristic colonies. When a short period of incubation is used, they are found to be among the most numerous organisms developing on the plate. Houston<sup>1</sup> found in 1898 four common spore-forming bacteria in the soil: *Bac. mycoides*, *Bac. subtilis*, a "granular bacillus," equivalent to *Bac. megatherium*, and *Bac. mesentericus* representing a group composed of a number of ill-defined, small spore-forming organisms.<sup>2</sup> Houston found *Bac. mycoides* to be present in the soil both in the vegetative stage and in the form of spores. Others,<sup>3</sup> however, demonstrated the spore-forming bacteria in the soil only in the spore stage.

The spores vegetate in the presence of a large amount of organic matter and an excess of moisture.<sup>4</sup>

A detailed study of the spore-forming bacteria has been made by various investigators,<sup>5</sup> the work of Ford<sup>6</sup> and associates being used as a basis for the following classification.

#### CLASSIFICATION OF SPORE-FORMING BACTERIA<sup>6</sup>

##### *Group I. Subtilis group*

Small, homogeneous, sluggishly motile organisms measuring 0.4 by 1.5 to 2.5 $\mu$ . No threads on glucose agar. Central or excentric spores, oval, measuring

<sup>1</sup> Houston, 1898 (p. 14).

<sup>2</sup> Conn, H. J. N. Y. Agr. Exp. Sta. Tech. Bul. 58. 1917.

<sup>3</sup> Conn, H. J. N. Y. Agr. Exp. Sta. Tech. Bul. 51. 1916.

<sup>4</sup> Winogradsky, S. Compt. Rend. Acad. Sci. 179: 861. 1924.

<sup>5</sup> Gottheil, O. Centrbl. Bakt. II, 7: 430-435, 449-465, 481-497, 529-544, 582-591, 627-637, 680-691, 717-730. 1901; Neide, E. Centrbl. Bakt. II, 12: 1-32, 161-176, 337-352, 539-554. 1904; Chester, F. D. Del Agr. Exp. Sta., Rept. 15: 42-96. 1904; Centrbl. Bakt. II, 13: 737-752. 1904; Holzmüller, K. Centrbl. Bakt. II, 23: 304-354. 1909; Conn. H. J. Jour. Inf. Dis. 46: 341-350. 1930.

<sup>6</sup> Ford, W. W., Lawrence, J. S., Laubach, C. A. and Rice, J. L. Jour. Bact. 1: 273-320, 493-534. 1916.

0.5 by 0.75 to 0.88 $\mu$ , often retaining terminal tags of protoplasm. Growth on solid media hard and penetrating, with tenacious scums on fluid media.

*Bacillus subtilis* Cohn

*Bacillus subtilis-viscosus* Chester

(Characterized by viscosity)

#### Group II. *Mesentericus* group

Small, homogeneous, actively motile organisms measuring 0.5 by 2 to 4 $\mu$ . They often produce long threads on glucose agar. The spores measure 0.5 by 1 to 1.12 $\mu$ , oval and retaining terminal tags of protoplasm. The growth on solid media is a soft pultaceous mass with tendency to wrinkle; on fluid media, growth is in the form of a friable easily-broken seum.

*Bacillus vulgatus* (Flügge) Trevisan

(*Bacillus mesentericus vulgatus* Flügge)

*Bacillus mesentericus* (Flügge) Migula

(*Bacillus mesentericus fuscus* Flügge)

*Bacillus atterrimus* Lehmann & Neumann

(*Bacillus mesentericus niger* Lunt)

*Bacillus globigii* Migula

(*Bacillus mesentericus ruber* Globig)

*Bacillus niger* Migula

(*Bacillus lactis niger* Gorini)

*Bacillus mesentericus* var. *flavus*

*Bacillus panis* Migula

(*Bacillus mesentericus panis viscosus* Vogel)

(Motility lost by capsule formation)

#### Group III. *Cohaerens-simplex* group

Motile organisms somewhat larger than either *Bacillus subtilis* or *Bacillus mesentericus*, measuring 0.37 to 0.75 by 0.75 to 3 $\mu$ . Thicker and longer forms on glucose agar. Involution and shadow forms are common and appear early. The spores are cylindrical, measuring 0.56 to 0.75 by 1 to 1.5 $\mu$ . A soft mass is formed on solid media; turbidity with little or no seum on liquid media.

*Bacillus cohaerens* Gottheil

*Bacillus simplex* Gottheil

*Bacillus agri* Ford and associates

*Bac. asteroides* and *Bac. teres* A. M. and Neide belong also to this group.

#### Group IV. *Mycoides* group

Large organisms with square ends growing in long chains. Single cells measure 0.5 by 3 to 6 $\mu$ . On glucose agar, the organisms are thicker and longer and are made up of globular bodies. Tendency for the organisms to grow in curves or spirals. The spores are central or excentric, round or oval to cylindrical, measuring 0.75 to 1 by 1 to 2  $\mu$ . Dry and penetrating growth on solid media; firm tenacious seum on liquid media.

*Bacillus mycoides* Flügge

*Bacillus prausnitzii* Trevisan

(*Bacillus ramosus liquefaciens* Prausnitz)

*Bacillus adhaerens* Ford and associates

(No motility)

*Group V. Cereus group*

Large, motile organisms with rounded ends, measuring 0.75 by 2.25 to 4 $\mu$ . Tend to grow in short chains. Thicker and longer on glucose agar, where protoplasm is converted into globular bodies. Central or excentric spores, cylindrical, measuring 0.5 to 0.75 by 1.12 to 1.5 $\mu$ . Spores retain protoplasm at one or both ends, often resembling enlarged *subtilis* or *mesentericus* spores. A soft pultaceous mass is formed on solid media, with tendency to fold or wrinkle; thick friable scum on liquid media.

*Bacillus cereus* Frankland (The *Bac. ellenbachensis* often referred to as an important soil organism belongs here).

*Bacillus albolactus* Migula

*Bacillus cereus* var. *fluorescens* Ford and associates

*Group VI. Megatherium group*

Very large, actively motile organisms, measuring 0.75 to 1.25 by 3 to 9 $\mu$ . Long forms are often produced; these spread out, lose their cytoplasm and show peculiar aggregations of protoplasm at the periphery. The protoplasm is rapidly converted into peculiar globular, highly refractile bodies, particularly on glucose agar. Shadow and transparent forms appear early. The spores are central, excentric or sub-terminal, oval to cylindrical, measuring usually 0.75 to 1.12 by 1.5 to 2 $\mu$ . Spores vary greatly in shape, being sometimes round, sometimes rectangular, often reniform. Growth on solid media as thick pultaceous mass, on liquid media as turbidity with little or no scum formation.

*Bacillus megatherium* De Bary

*Bacillus pastasites* Gottheil

*Bacillus ruminatus* Gottheil

*Group VII. Round terminal spored group*

Small, actively motile organisms, measuring 0.5 to 0.75 by 1.5 to 3 $\mu$ , often forming long threads in old cultures. Protoplasm homogeneous. Spores sub-terminal or terminal, round, thicker than the organisms from which they spring, measuring 1 to 1.5 $\mu$  in diameter.

*Bacillus pseudotetanicus* (Kruse) Migula

(*Bacillus pseudotetanicus* var. *aerobius* Kruse)

*Bacillus fusiformis* Gottheil

*Group VIII. Cylindrical terminal spored group*

Small, thin, actively motile organisms, measuring 0.37 to 0.5 by 2.5 to 4 $\mu$ . Slightly larger on glucose agar but no change in character of protoplasm. Spores terminal, cylindrical, measuring usually 0.75 by 1.12 to 1.5 $\mu$ .

*Bacillus circulans* Jordan

*Bacillus brevis* Migula

*Bacillus terminalis* Migula

*Group IX. Central spored group*

Long, actively motile organisms with pointed ends, measuring 0.37 to 0.5 by 1.12 to 4 $\mu$ . Slightly larger on glucose agar, but no change in character of proto-

plasm. The spores develop in the middle of the rods, which become spindle-shaped. The spores are large, cylindrical, measuring 0.6 to 0.8 by 1.12 to 1.5 $\mu$ .

*Bacillus centrosporus* Ford and associates

*Bacillus laterosporus* Ford and associates

A summary of the characteristic points of the spore-forming bacteria, recognized by A. Meyer and his associates is given in table 16.<sup>7</sup>

*Occurrence of aerobic, spore-forming bacteria in the soil.* By the use of gelatin plates the three most common spore-forming bacteria in the soil can be readily recognized. Except for the non-spore forming *Bact. fluorescens*, *Bac. mycoides* is the most rapid liquefier; it produces large filamentous to rhizoid colonies. *Bac. cereus* liquefies gelatin almost as rapidly as *Bac. mycoides* and usually forms round colonies with entire edges; the surface membrane contains granules which tend to be arranged concentrically. *Bac. megatherium* liquefies gelatin more slowly; its colonies are seldom over 1 cm. in diameter and are characterized by a flocculent center composed of white opaque granules, surrounded by a zone of clear liquefied gelatin; the smaller colonies have no surrounding zone and are recognized only by their granular structure.

These organisms as well as various other spore-forming bacteria are of universal occurrence in the soil. The following forms were demonstrated<sup>8</sup> in the soil of Northern Greenland: *Bac. subtilis*, *Bac. mesentericus*, *Bac. asterosporus*, and *Bac. malabarensis*. According to Flüggé<sup>9</sup> *Bac. mycoides* is present in almost every soil examined. Holzmüller<sup>10</sup> also found one form or another of *Bac. mycoides* in every soil. Gottheil found *Bac. asterosporus* as well as *Bac. ellenbachensis* very abundantly in the soil. *Bac. cohaerens*, *Bac. fusiformis*, *Bac. petasites*, *Bac. graveolens*, *Bac. pumilus*, *Bac. ruminatus*, *Bac. subtilis* and *Bac. tumescens* occurred to a less extent. *Bac. simplex* was found only once, while the presence of *Bac. mycoides* and *Bac. carotarum* could not be established.

According to Ford and associates, *Bac. cereus* is very predominant in Maryland soils, followed by *Bac. subtilis*; *Bac. mesentericus* was more abundant than *Bac. vulgatus*, while *Bac. megatherium*, *Bac. petasites* and *Bac. mycoides* were found only rarely. Five hundred and twenty

<sup>7</sup> Stapp, C. Centrbl. Bakt. II, 61: 1-71. 1920.

<sup>8</sup> Barthel, Ch. Saertryk Meddel. Groenland 64, Kopenhagen. 1922.

<sup>9</sup> Flüggé, C. Leipzig. 1896.

<sup>10</sup> Holzmüller, 1909 (p. 138). Some of the other bacteria described by this investigator may be merely stages in the life cycle of *Bac. mycoides*, as shown by Oesterle, P. and Stahl, C. A. Centrbl. Bakt. II, 79: 1-25. 1929.



cultures were isolated from eight soils (five near Baltimore and three from Nazareth, Pa.). All soil samples were boiled in water for 20 minutes just before plating, to kill all the vegetative cells of bacteria as well as the other soil organisms and leave only the spores of the spore-forming bacteria. This is brought out in the following summary:

ORGANISM	PRESENCE IN NUMBER OF SOIL SAMPLES	
	Baltimore soil	Nazareth soil
<i>Bac. petasites</i> .....	73	116
<i>Bac. cereus</i> .....	134	41
<i>Bac. megatherium</i> .....	29	13
<i>Bac. subtilis</i> .....	24	9
<i>Bac. mesentericus</i> .....	9	11
<i>Bac. vulgatus</i> .....	12	6
<i>Bac. mycoides</i> .....	15	2
<i>Bac. mesentericus</i> var. <i>flavus</i> .....		9
<i>Bac. cereus</i> var. <i>fluorescens</i> .....	3	
<i>Bac. fusiformis</i> .....	3	2
<i>Bac. brevis</i> .....		3
<i>Bac. simplex</i> .....	1	
<i>Bac. cohaerens</i> .....	1	2
<i>Bac. agri</i> .....	2	
Total isolations.....	306	214

The three most common types of spore-forming bacteria found in the soil by Conn were: *Bac. megatherium* (averaging about 375,000 per gram of soil), *Bac. mycoides* (225,000 per gram) and *Bac. cereus* (180,000 per gram). The relative number of spore-forming bacteria in the soil depends on the length of the incubation period. When a short incubation period is used, nearly half of the colonies on the plate may be found to consist of spore-forming organisms.<sup>11</sup> When a long incubation period is used they are found to form only about 5 per cent of the colonies.<sup>12</sup> The presence of spore-forming bacteria, which are destroyed with difficulty by partial sterilization, varies with the soil.<sup>13</sup> Here belong *Bac. mycoides*, *Bac. mesentericus* and *Bac. mesentericus ruber*. The simplest way of destroying these is to heat the soil for 30 minutes at 100°C. on seven

<sup>11</sup> Chester, F. D. Del. Agr. Exp. Sta., Rept. 14: 52-63. 1903.

<sup>12</sup> Hiltner and Störmer, 1903 (p. 12); Conn, 1917 (p. 138).

<sup>13</sup> Eckelmann, E. Centrbl. Bakt. II, 48: 140. 1918.

consecutive days and then to incubate the soil, between successive sterilizations, at room temperature.

Various groups of bacteria are represented in the soil by forms which are distinct in some of their physiological characteristics from the same organisms found on other substrates, as in the case of the lactobacilli; the soil forms of this group are not able to ferment lactose.<sup>14</sup>

*Non-spore forming bacteria.* Although the heterotrophic non-spore forming bacteria include the most predominant group of soil organisms developing on the plate and even more so in the microscopic examination of the soil, very little attention has been paid to their identification. This has been due, probably, to the fact that most of these bacteria grow poorly and only very slowly on laboratory culture media. The inappreciable results obtained by the ordinary chemical tests led to the general assumption that they are of little importance in the soil. The fact must also be kept in mind that certain very important physiological groups of soil bacteria such as the *Thiobacillus* group, the nitrite, nitrate forming and other autotrophic bacteria, as well as various heterotrophic bacteria, such as many of the aerobic cellulose decomposing bacteria, certain urea bacteria, etc., belong to this group of organisms.

Except for the physiological groups just mentioned, the study of the heterotrophic, non-spore-forming bacteria, which do or do not develop on the common synthetic and nutrient media, has been neglected. This has been largely a result of the lack of proper methods of study. Organisms have been looked for which take part in the various known processes, largely in the nitrogen transformation in the soil. If an organism did not take part in the processes of nitrification or sulfur-oxidation, nitrogen-fixation or cellulose decomposition, and if it did not produce ammonia rapidly from proteins, it was assumed to be unimportant in the soil.

We must assume from the meager information available that the non-spore-forming bacteria take part in the slow but constant decomposition of the soil organic matter. Hiltner and Störmer referred to them as the non-liquefying group of bacteria and found them to be by far the largest group of soil organisms; they develop abundantly after certain treatments of soil, which are decidedly beneficial in nature. Conn referred to these organisms as the "slow-growing" group of bacteria, and also found them to be of the greatest abundance and universal occurrence in the soil. Winogradsky considered these bacteria as forming the native (*autochthonous*) population of the soil. When

<sup>14</sup> Hunt, G. A. and Rettger, L. F. Jour. Bact. 20: 61-83. 1930.

fresh organic matter is added to the soil, the fungi and the spore-forming bacteria at once become active, until the organic matter is reduced to a certain consistency, namely the humus stage. These organisms then become inactive again until a fresh supply of energy is introduced into the soil. The humus left is constantly acted upon by the non-spore-forming bacteria (and actinomyces), which mineralize it, liberating the nitrogen and other mineral elements into available forms.

*Classification.* The heterotrophic, non-spore-forming, aerobic bacteria usually produce punctiform colonies on agar and gelatin, are chromogenic or non-chromogenic, motile or non-motile, some liquefy gelatin rapidly, while others only slowly or not at all. The most important representatives of the group of rapid-liquefying organisms is the *Bact. fluorescens* or *Pseud. fluorescens*. The whole group is often spoken of as the *fluorescens* group, although many of the organisms never produce any fluorescence.

The representatives of the slow-liquefying group of bacteria form pinpoint colonies on the agar and gelatin plate and are characterized by poor growth in all liquid media and by the formation of punctiform colonies on gelatin. These organisms are usually less than one micron in length and less than half a micron in diameter, being short rods or cocci; growth on agar streaks is fair, soft, smooth, glistening, slimy to watery. Due to the fact that they grow only poorly in liquid media, they do not lend themselves readily to physiological studies.<sup>1</sup> They also appear much the same morphologically. The silica gel plate with soil extract as the nutritive ingredient presents a new and promising method for their study.

Conn<sup>15</sup> divided these organisms into five groups on the basis of their growth upon a synthetic medium (1.0 gram  $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.2 gram KCl, 0.2 gram  $\text{MgSO}_4$ , 10.0 sugar in 100 cc. of water, adjusted to pH 7.0):

1. Organisms forming small short rods, usually under 0.5 micron in diameter, non-motile or having one or possibly two polar flagella; no tendency to change in morphology but very variable in their physiology, such as liquefaction of gelatin and gas formation from nitrate. *Bact. parvulum* Conn represents one of the types belonging to this group.

2. Organisms that appear for a day or two after inoculation on a new medium as small short rods, less than  $0.5\mu$  in diameter, then shorten and appear like micrococci. All liquefy gelatin, slowly. *Bact. globiforme* Conn can be considered as a representative of this group; it is

<sup>15</sup> Conn, H. J. N. Y. Agr. Exp. Sta. Tech. Bul. 115. 1925; Centrbl. Bakt. II, 76: 65-88. 1928; Tech. Bul. 128, N. Y. Agr. Exp. Sta. 1928.

most abundant in productive soils; its presence in soil was believed to be a good index of the availability of the soil nitrogen.

3. Small short rods, with a tendency to produce long filaments, usually unbranched, but frequently branched.

4. Organisms consisting, in young cultures, mostly of branching forms, apparently produced by the germination of small spherical arthrospores. The branching forms disappear in a few days, leaving the coccoid forms.

5. Organisms occurring normally as cocci, but with a tendency to produce rods and filaments after a few days of growth on ordinary media. This group is more abundant in manure than in soil.

The first two groups are most numerous in the soil. According to Winogradsky<sup>16</sup> the cocci predominate, as shown by the direct microscopic method (including probably also the short rods). These organisms were found to grow in groups (masses, zooglea) imbedded in the colloids which cover the soil particles.

The term "*Micrococcus*" was applied<sup>17</sup> to designate the genus of irregular mass-forming cocci. Sixteen species were separated on the basis of gelatin liquefaction, nitrate reduction and ability to utilize certain ammonium salts as the only source of nitrogen.

The fact must also be mentioned that a few spore-forming organisms are found to form punctiform colonies. They are not related to the other spore-forming bacteria in their general physiology and stand also apart from the "slow growers." Except the *Bact. fluorescens* and *Bact. caudatum*, the other non-spore-forming bacteria cannot be readily recognized by their colonies on gelatin or agar. The rapid liquefaction of the gelatin by the former and the orange color of the latter allow a ready recognition of those two types. *Bact. aerogenes* and *Bact. coli* isolated from the soil can be readily distinguished from *Bact. coli* of fecal origin by the fact that the former use citrates as a source of carbon and the latter does not; also by the production of a red iron rust growth on Harder's medium.<sup>18</sup>

*Occurrence of non-spore-forming bacteria in the soil.* The non-spore-forming bacteria are numerically the largest group of soil microorganisms. It still remains to be determined what bulk they occupy in the soil population and what relative importance may be ascribed to them

<sup>16</sup> Winogradsky, S. Ann. Inst. Past. 39: 299-354. 1925.

<sup>17</sup> Hucker, G. J. N. Y. Agr. Exp. Sta. Tech. Bul. 99, 1924; 100, 1924; 101, 1924; 102, 1924; 135, 1928.

<sup>18</sup> Koser, S. A. Jour. Bact. 8: 493-520. 1923; 9: 59-77. 1924; Murray, T. J. and Skinner, C. E. Proc. Soc. Exp. Biol. Med. 23: 104-106. 1925.

in the transformations which take place in the soil. Seventy-five per cent of the total number of colonies developing on the plate are non-spore-forming bacteria and cocci; the other 25 per cent include the spore-forming organisms and actinomyces. Those colonies are made up largely of the slow growing organisms; between 2 to 60 millions of these bacteria, as determined by the plate method, are found per 1 gram of soil. The non-spore-forming bacteria are believed to be very active in the soil, particularly in view of their great variability as affected by the soil treatment. Conn found that a certain soil contained 350,000 rapid-liquefying colonies, 11,000,000 punctiform colonies and 4,700,000 spore-formers and actinomyces, before aeration; after aeration for one day these numbers changed to 1,500,000 of the first, 22,500,000 of the second, and 4,000,000 of the third. Aeration of soil and the addition of organic matter bring about the greatest numerical increase in the group of non-spore-forming bacteria in the soil. The above numbers as determined by the plate method represent only a fraction of the actual abundance of these organisms in the soil, since they live in zooglea-like masses imbedded in the soil colloids and cannot be readily separated into individual cells.

Of the non-spore-forming bacteria and cocci, some are especially abundant in the soil. The available information on this subject is very meager due largely to the difficulty of studying some of the slow growing organisms on artificial media. The *Bact. fluorescens* is especially abundant, having been found in soils all over the world. The same is true of certain cocci or very short rods.

Severin<sup>19</sup> found rods to predominate in freshly plowed manured soil as well as in manure itself; however, in two weeks cocci predominated. According to Houston,<sup>20</sup> *Bact. vulgare* (*Proteus vulgaris*), *Bact. prodigiosum* and various *Sarcinae* are less abundant in the soil than the spore-forming bacteria, actinomyces, and the *Bact. fluorescens*. When inoculated into soil, *Bact. prodigiosum* persisted only when the soil was previously sterilized and kept moist; when the soil was air dried or when the inoculation took place into non-sterile soil, the organism was rapidly destroyed. The occurrence of brown fluorescent bacteria in the soil was pointed out by Bazarewski.<sup>21</sup> Barthel<sup>22</sup> found in a North Greenland soil

<sup>19</sup> Severin, S. A. Centrbl. Bakt. II, 1: 100-104. 1895; Zhur. Opit. Agron. 1: 463-489. 1900.

<sup>20</sup> Houston, 1898 (p. 14).

<sup>21</sup> Bazarewski, S. v. Centrbl. Bakt. II, 15: 1-7. 1905; see also C. Hüttig. Ber. deut. bot. Gesell. 47: 395-400. 1929.

<sup>22</sup> Barthel, 1922 (p. 141).

*Bact. fluorescens*, *Bact. caudatum*, *Bact. punctatum*, *Bact. violaceum*, *Bact. lactis viscosum*, *Bact. umbilicatum*, *Bact. ochraceum* and *Bact. zopfii*. Among the cocci, Barthel found *Tetracoccus* and *Sarcina flava*. In addition to these organisms, various other bacteria, like those forming a blue pigment, those accompanying the nitrogen-fixing organisms, various spirilli, vibrios, and cocci (*Streptococcus pyogenes*, *Strep. acidi lactici*, *Sarcina lutea*, *S. aurantiaca*) have been reported to be found in the soil by different investigators. Certain organisms, like *Bact. vulgare*, *Bact. coli* and others, present extensively in manure and feces, are thus abundantly introduced into the soil and may survive there for a long time; some of those, such as various proteolytic and cellulose-decomposing bacteria, may even become active there.

The bacteria of the so-called colon group found abundantly in the feces of warm blooded animals differ, in certain cultural characteristics,<sup>23</sup> from those found in the soil, the former being largely *Bact. coli* and the latter belonging to the *Bact. aerogenes* group, which is even capable of fixing small amounts of nitrogen. Out of 467 strains of bacteria isolated from various soils by Chen and Rettger, 430 were identified as *Bact. aerogenes*, 17 as *Bact. cloacae* and only 20 as *Bact. coli*;<sup>9</sup> the sources of the *Bact. coli* strain were shown by a sanitary survey to be not entirely free from animal pollution. However, all of 173 organisms found in the feces of various animals were typical *Bact. coli*. When *Bact. coli* and even *Bact. aerogenes* are added to the soil in considerable numbers, they rapidly die out,<sup>24</sup> since conditions are not very favorable for their development, although *Bact. coli* was found to survive in the soil for 4 years.

It is of interest to note here that Stoklasa<sup>25</sup> found the following organisms in the "rhizosphere" or in close proximity to the root system of plants, consisting largely of non-spore-forming bacteria:

*Bact. acidi lactici*  $\alpha$  and  $\beta$   
*Bact. pneumoniae*  
*Bact. coli* group  
*Bact. proteus vulgaris*  
*Bact. ochraceum*  
*Bact. fulvum*  
*Bact. punctatum*

*Bact. putidum*  
*Bact. fluorescens liquefaciens*  
*Bact. anthracoides*  
*Azotobacter chroococcum*  
*Butyric acid bacteria*  
*Bac. mycoides*  
*Bac. mesentericus*

<sup>23</sup> Chen, C. C. and Rettger, L. F. Jour. Bact. 5: 253-298. 1920; Levine, M. Iowa Engin. Exp. Sta. Bul. 62. 1921.

<sup>24</sup> Skinner, C. E. and Murray, T. J. Jour. Inf. Dis. 38: 37-41. 1926; Young, C. C. and Greenfield, M. Amer. Jour. Publ. Health, 13: 270-273. 1923; Koser, S. A. Jour. Amer. Water Works Assn. 15: 641-646. 1926.

<sup>25</sup> Stoklasa and Doerell, 1926 (p. xiv).

*Thermophilic bacteria.* Miquel<sup>26</sup> was the first to isolate, in 1879, bacteria capable of developing at 72°C. These organisms were found not only in river mud, sewage excreta, dust, but also in soil. Globig,<sup>27</sup> Macfayden and Blaxall<sup>28</sup> and others<sup>29</sup> soon established that the soil harbors various bacteria which are capable of growing at 50° to 70°C., but refuse to grow in the laboratory at room temperature. Organisms capable of growing at temperatures up to 79.5°C. were also found<sup>30</sup> in stable manure. Schloesing<sup>31</sup> demonstrated in 1892 that the self-heating and burning of hay, cotton, manure, etc., is caused by microorganisms which he called thermogenic bacteria. It may be here largely a question of thermotolerant organisms rather than of strict thermophiles.<sup>32</sup> Thermophilic bacteria are found in the sands of the Sahara Desert,<sup>33</sup> but are absent in forest soils. The distribution of these organisms in the soil seems to depend on the manure used. One to ten per cent of the flora of garden soils heavily manured, developing on the plate, may consist of thermophilic forms, while field soils contain only 0.25 to 0 per cent of these bacteria. Uncultivated soils may be entirely free from thermophilic organisms, the geographic condition having no influence.<sup>34</sup>

Globig isolated a number of thermophilic bacteria from the soil and believed that, because most of them were isolated from the surface layers, the rays of the sun supply the heat necessary to obtain the high temperatures. Rabinowitsch<sup>29</sup> isolated from soil and feces eight species of thermophilic bacteria, varying in morphology (rod shaped to comma shaped), size of spores, color of colony on agar and potato. A number

<sup>26</sup> Miquel, P. *Ann. Microg.* 1: 4-10. 1888; see Tsiklinski. *Ann. Inst. Past.* 13: 505. 1899.

<sup>27</sup> Globig. *Ztschr. Hyg.* 3: 294-321. 1888.

<sup>28</sup> Macfayden, A. and Blaxall, F. R. *Jour. Path. Bact.* 3: 87-99. 1894.

<sup>29</sup> Rabinowitsch, L. *Ztschr. Hyg.* 20: 154. 1895; Oprescu, V. *Arch. Hyg.* 33: 164. 1898; Schillinger, A. *Hyg. Rundsch.* 1898, 568; Tsiklinski, P. *Russ. Arch. Pathol.* 5, 1898; (Ambroz, A. *Centrbl. Bakt.* I, Ref. 48: 257-279, 289-312. 1910); Sames, T. *Ztschr. Hyg.* 33: 313. 1910; Blau, O. *Centrbl. Bakt.* II, 15: 97-143. 1906; Krohn, V. *Suom. Tied. Toim. A.*, 21: 125, 1923; Feirer, A. *Soil Sci.* 23: 47-56. 1927.

<sup>30</sup> Burrill, T. J. *Ill. Agr. Exp. Sta. Bul.* 7. 1889; Schloesing, Th. *Compt. Rend. Acad. Sci.* 109: 835-840. 1889; Dupont, C. *Ann. Agron.* 28: 289. 1902.

<sup>31</sup> Schloesing, T. *Ann. Agron.* 18: 5. 1892.

<sup>32</sup> Miehe, H. *Die Selbsterhitzung des Heus.* G. Fischer. Jena. 1907; Schütze, H. *Arch. Hyg.* 67: 35-56. 1908; *Centrbl. Bakt.* II, 71: 440-490. 1927.

<sup>33</sup> Nègre, L. *Compt. Rend. Soc. Biol.* 74: 814-816. 1913.

<sup>34</sup> Migula, W. *Forstwiss. Zentrbl.* 57 (N. F. 35): 161-169. 1913; Mischustin, E. *Centrbl. Bakt.* II, 66: 328-344. 1926; 71: 416-433, 1927.

of other bacteria were isolated which had an optimum at 60° to 65°C. and were killed by heating at 100°C. for 8 to 20 hours. A variety of *B. coli* and a spore-bearing organism, *Bac. calfactor*, as well as several fungi and actinomyces, were found capable of growing at high temperatures and taking an active part in the decomposition of hay.<sup>32</sup> The size of *Bac. calfactor* changes with temperature at which it is grown (on hay infusion agar at 70°C. it is 5 to 0.4 $\mu$ ; at 56°C.—5 by 0.7 $\mu$ ; at 30°C.—3 by 0.8 $\mu$ ). The rods occur singly, not in chains, and are motile at favorable temperatures (above 30°). The spores are 1.5 by 0.8 $\mu$ . Spore germination takes place in 60 minutes at 60°C.; in 85 minutes, 2 cells are already formed. This rapid multiplication accounts for the fact that at 50°C., turbidity is definite in liquid decoctions within six hours and an abundant growth is produced on agar.

De Kruffyff<sup>35</sup> isolated from the soil ten species of rod shaped, thermophilic bacteria, largely long rods forming oval to round spores; most of them produce proteolytic enzymes and do not grow on potato. He suggested that with a rise in temperature in tropical soil they take the place of the ordinary bacteria. It has been established that there occur in soils thermophilic nitrogen-fixing bacteria,<sup>36</sup> thermophilic cellulose decomposing bacteria<sup>37</sup> and thermophilic denitrifying bacteria.<sup>38</sup> The nature of the medium was found<sup>39</sup> to have an important influence upon the temperature optimum of the organism. Two per cent of glucose is added to soil either alone or with 2 per cent CaCO<sub>3</sub>; this is then steamed at 100° for 20 minutes, to kill the non-spore-forming organisms. Water is added to bring the moisture to 16 per cent and the soils are incubated at 52°C. for 4 to 6 days, at which time the bacteria are isolated. These bacteria grow in media at 52°C., but not at 28° to 30°; when reinoculated into soil, they may grow well even at 15° to 20° although not so abundantly. Miede assumed that these organisms can develop only in manure heaps where a great deal of heat is generated. However, others<sup>39</sup> suggested that they do not always lead a latent life in the soil, but find in summer a suitable temperature for their development.

<sup>35</sup> de Kruffyff, E. Centrbl. Bakt. II, 26: 65-74. 1910.

<sup>36</sup> Pringsheim, 1911 (p. 116).

<sup>37</sup> Pringsheim, 1913 (p. 195); Kroulik, A. Centrbl. Bakt. II, 36: 339-346. 1912; Viljoen, Fred and Peterson, 1926 (p. 195).

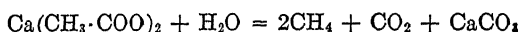
<sup>38</sup> Ambroz, A. Centrbl. Bakt. II, 37: 3-16. 1913.

<sup>39</sup> Koch, A. and Hoffmann, C. Centrbl. Bakt. II, 31: 433-436. 1911; Krohn, V. Ann. Acad. Sci. Fennicae, Ser. A. 21: 1-125. 1923.



Coolhaas<sup>40</sup> isolated from soil and manure two thermophilic bacteria both capable of decomposing starches: *Bac. thermoamylolyticus* produced, at 55°C, a large amount of maltose, very little acid and no gas, while the facultative anaerobic *Bac. thermobutyricus* produced carbon dioxide and hydrogen, as well as a mixture of organic acids, largely butyric. The presence of thermophilic lactic acid producing bacteria (*Thermobacillus tarbellicus*) in soil and in manure has been established by Guittonneau.<sup>41</sup>

Among the processes carried out by thermophilic bacteria, the decomposition of organic matter, especially of celluloses, received considerable attention (p. 195). Many of the thermophilic bacteria are strongly proteolytic. Some decompose fatty acids, with the formation of methane and carbon dioxide, the ratio of the former to the latter being 2:1, in the case of some acids, and 1:1 in the case of formic.



Cane sugar is also fermented to methane and carbon dioxide, probably through the acetic or formic acid stages.

Most of the thermophilic bacteria are spore-forming aerobes. Some are spore-forming facultative anaerobes. Some of them are facultative thermophilic, since they can grow at 20°, have their optimum at 50°C. and maximum at about 60°C.<sup>42</sup>

*Mycobacteria.* The Mycobacteria are a group of organisms which differ from true bacteria by the formation of more or less long monodially branched threads under normal conditions of growth. They are largely acid fast and represent botanically a well defined group of organisms, standing midway between the true bacteria and the actinomyces group. In stained preparations, in culture upon artificial media, as well as in a number of their physiological activities, the mycobacteria resemble actinomyces.

A number of mycobacteria were isolated from manure<sup>43</sup> and from soil.<sup>44</sup> A selective development of these organisms will take place upon agar

<sup>40</sup> Coolhaas, C. Centrbl. Bakt. II, 75: 161-170, 344-360. 1928; 76: 38-44. 1928.

<sup>41</sup> Guittonneau, G. Compt. Rend. Acad. Sci. 187: 69-72. 1928.

<sup>42</sup> Bergey, D. H. Jour. Bact. 4: 301-306. 1919. A recent review of the literature on thermophilic bacteria is given by A. H. Robertson. Tech. Bull. 130, N. Y. Agr. Exp. Sta. 1927.

<sup>43</sup> Moëller, A. Centrbl. Bakt. 25: 369-373. 1899.

<sup>44</sup> Söhngen, 1913 (p. 153); Vierling, K. Diss. Univ. Heidelberg. 1921.

plates, containing the necessary minerals, an inorganic source of nitrogen and inoculated with a soil suspension, if the cultures are placed under a bell jar together with a dish of benzene or petroleum; paraffin coated pebbles may be used as a source of carbon.<sup>45</sup> The incubation temperature is usually 30°C.; in order to avoid fungus development, 47.5°C. may be employed. The final isolation and purification of the organisms can be accomplished by means of ordinary bacteriological methods. Spore formation takes place by contraction of the cell contents, in a manner similar to that of the actinomyces, giving coccus-like fragments. The nature of these spores is, therefore, quite distinct from that of bacterial endospores. The colonies on solid substrates have a certain thread-like structure (No. 45, Pl. VI).

Four definite species of mycobacteria were at first recognized,<sup>46</sup> namely *M. lacticola*, *M. phlei*, *M. luteum* and *M. eos*, which is frequently considered as a variety of *M. lacticola*. The latter organism shows various morphological modifications which may occur spontaneously and which can be produced experimentally. Gray and Thornton<sup>46</sup> divided the mycobacteria into two genera: *Mycoplana*, with flagella, and *Mycobacterium*, without flagella. Two species of the first genus and six species of the second were described in detail. One species, *Mycobact. agreste*, was found to be more abundant in dry than in wet soils.

The mycobacteria readily utilize as sources of energy glycerol, various lower fatty acids, waxes, hydrocarbons and aromatic compounds.<sup>47</sup> Inorganic nitrogen sources seem to be preferred. Most of them reduce nitrates to nitrites. Their optimum temperature lies between 22° and 37°C. and their optimum reaction at pH 6.4 to 7.5. They form pigments readily. Their importance in soil processes seems to consist in the decomposition of various organic complexes, such as hydrocarbons and aromatic compounds.

*Myxobacteria*.<sup>48</sup> Morphologically the Myxobacteria represent a special group of organisms, quite distinct from the plasmodium-producing *Myxomycetes* and the pseudo-plasmodium forming *Acrasidae*. They are even considered by some investigators<sup>49</sup> as colorless blue algae.

<sup>45</sup> Frey, C. A. Science, 71: 366. 1930; Büttner, H. Arch. Hyg. 97: 12. 1926.

<sup>46</sup> Haag, F. E. Centrbl. Bakt. II, 71: 1-45. 1927; Gray, P. H. H. and Thornton, H. G. Centrbl. Bakt. II, 73: 74-96. 1928.

<sup>47</sup> Söhngen, N. L. Centrbl. Bakt. II, 37: 595-609. 1913.

<sup>48</sup> Thaxter, R. Bot. Gaz. 14: 389. 1902; 23: 395. 1897; 37: 405. 1904; Quehl, A. Centrbl. Bakt. II, 16: 9-34. 1906.

<sup>49</sup> John, E. Beiträge zur botanischen Protistologie. I. Die Polyangiden. Leipzig. 1924.

Myxobacteria occur abundantly in manure and in soil, the total number of organisms and species depending upon the nature of the soil. Some forms are found only in alkaline, neutral or faintly acid (pH 8.0–6.0) soils; others are found in very acid soils (pH 3.7–5.9); still others occur in soils of various reactions. Moist soils are more favorable for their development than dry soils; cultivation of soil is also favorable. Peat bogs and moist forest soils contain a specific flora of these organisms.<sup>50</sup> To demonstrate the presence of Myxobacteria in soil, balls of rabbit manure, previously moistened with water and sterilized in the autoclave, are placed on the surface of the soil; as many as 7 to 10 species may thus be obtained from a single sample of soil.

*Bacteria reducing nitrates to nitrites and ammonia.* A large number of organisms are able to reduce nitrates to nitrites or to ammonia. However, only specific bacteria are capable, under certain conditions, of reducing the nitrate and the nitrite to elementary nitrogen and oxides of nitrogen, in which form the nitrogen escapes into the atmosphere. Under anaerobic conditions, the nitrate and nitrite may serve as sources of oxygen for these bacteria, which enables them to oxidize the available sources of energy.<sup>51</sup> The last process is usually referred to as complete or direct denitrification and the bacteria concerned in this process are spoken of as *denitrifying bacteria*.

The presence of organisms in soil capable of reducing nitrates to nitrites was first demonstrated by Schönbein<sup>52</sup> in 1868, then by others especially by Gayon and Dupetit.<sup>53</sup> In addition to various bacteria,<sup>54</sup> certain yeasts, filamentous fungi, and actinomycetes<sup>55</sup> take an active part in this process. Frankland<sup>56</sup> called attention to the fact that the reduction of nitrate by certain bacteria (*Bac. ramosus* and *Bac. pestifer*) is favorably influenced by increasing the organic matter content of

<sup>50</sup> Krzemieniewski, H. and S. Acta Soc. Bot. Poloniae, 4: 1–54. 1926; 5: 1–20, 102–139. 1927.

<sup>51</sup> Weissenberg, H. Arch. Hyg. 30: 279–290. 1897; Jensen, H. Centrbl. Bakt. II, 3: 622–627, 689–698. 1897; 4: 401–411, 449–460. 1898; Pakes, W. C. C., and Jollyman, W. H. Jour. Chem. Soc. I, 79: 322–329. 1901.

<sup>52</sup> Schönbein, C. F. Jour. prakt. Chem. 105: 208–214. 1868.

<sup>53</sup> Gayon, U., and Dupetit, G. Compt. Rend. Acad. Sci. 95: 644–646, 1365–1367, 1882; Recherches sur la reduction des nitrates par les infiniments petits. Nancy. 1886; Mem. Soc. Sci. phys. Nat. Bordeaux. 1886; Ann. Sci. Agron. 1: 226–325. (1885) 1886.

<sup>54</sup> Maassen, A. Arb. K. Gesundheitsamt, 18: 21–27. 1901.

<sup>55</sup> Wolff, K. Hyg. Rundschau, 91: 538. 1899; Waksman, 1919 (p. 276).

<sup>56</sup> Frankland, P. J. Chem. News, 57: 89. 1888; Ztschr. Hyg. 6: 373. 1899.

the solution, especially the peptone. Anaerobiosis or lack of sufficient aeration also favors nitrite formation.<sup>57</sup> Nitrite-forming bacteria are well distributed in the soil.<sup>58</sup>

Out of 109 species of bacteria tested by Maassen, in a solution containing 5 per cent peptone and 0.5 per cent sodium nitrate, 85 reduced nitrates to nitrites, especially *Bact. pyocyaneum*; 46 reduced the nitrate to ammonia and 4 liberated atmospheric nitrogen. Out of 28 species of bacteria studied by Klaeser, all but one were found capable of reducing nitrates. Many strict aerobic bacteria can live anaerobically in the presence of nitrates. Intensive aeration inhibits the process of nitrate reduction. The reaction of the medium has an important influence in determining whether nitrates are reduced to nitrites or ammonia; an alkaline reaction favors the first process and an acid reaction the second.

Klaeser used a medium having the following composition:

KNO <sub>3</sub> .....	2 grams	NaCl.....	0.1 gram
Glucose.....	10 grams	MgSO <sub>4</sub> .....	0.3 gram
K <sub>2</sub> HPO <sub>4</sub> .....	1 gram	FeCl <sub>3</sub> .....	0.01 gram
CaCl <sub>2</sub> .....	0.1 gram		

Other media, with and without peptone, but containing nitrates, can also be used for demonstrating nitrate reduction by bacteria.

The following organisms can be recorded as capable of reducing nitrates to nitrites: *Bact. coli*, *Bact. vulgare* and allied species,<sup>59</sup> *Bact. prodigiosum*, *Bact. putidum*, *Bact. fluorescens*, *Bact. pyocyaneum*, *Bact. herbicola*, *Bac. subtilis* and allied species, *Bac. vulgatus*, *Bac. mycoides*, *Micr. pyogenes*, *Mycobact. phlei* and other mycobacteria, *B. porticensis* and others. Some of these organisms, such as *Bact. coli*, are also capable of liberating hydrogen.<sup>60</sup> The products formed from the reduction of the nitrate depend largely upon the composition of the medium and oxygen tension.

Marchal<sup>61</sup> demonstrated that certain bacteria (*Bac. mycoides*) reduce nitrates to ammonia, with the intermediate formation of nitrites. According to Beijerinck and van Delden<sup>62</sup> various bacteria, like *Bac. sub-*

<sup>57</sup> Laurent, E. Ann. Inst. Past. 4: 722-744. 1890; Kühl, H. Centrbl. Bakt. II, 20: 258-261. 1908; Caron, H. V. Centrbl. Bakt. II, 33: 62-116. 1912.

<sup>58</sup> Jensen, 1897 (p. 154); Klaeser, M. Centrbl. Bakt. II, 41: 365-430. 1914; Ber. deut. bot. Gesell. 32: 58. 1914.

<sup>59</sup> Horowitz, A. Ann. Inst. Past. 30: 307-318. 1916.

<sup>60</sup> Mazé, P. Ann. Inst. Past. 25: 289-312, 369-391. 1911.

<sup>61</sup> Marchal, E. Agr. Sci. 8: 574. 1894; Centrbl. Bakt. II, 1: 758. 1895.

<sup>62</sup> Beijerinck, M. W. and van Delden, A. Centrbl. Bakt. II, 9: 3-43. 1902; Stoklasa, J. and Vitek, E. Centrbl. Bakt. II, 14: 102-118. 1905.

*tilis* and *Bac. mesentericus vulgatus*, are capable of producing both ammonia and nitrite from nitrates, but no ammonia from nitrites; *Azotobacter chroococcum*, however, produced ammonia from nitrates and nitrites. The reduction process takes place in the presence of carbohydrates and organic acids as sources of carbon. These bacteria undoubtedly include the "protein-forming bacteria" described by Gerlach and Vogel,<sup>63</sup> capable of transforming nitrate into protein nitrogen with an intermediate reduction to ammonia nitrogen.

*Bacteria reducing nitrates to atmospheric nitrogen.* The formation of gaseous nitrogen in the process of decomposition of organic matter in the soil was first observed by Davy.<sup>64</sup> This was ascribed to a chemical interaction between nitrites and amino acids in the soil, resulting in the formation of gaseous nitrogen.<sup>65</sup> Gayon and Dupetit pointed out in 1882 that bacteria were responsible for this process and that the free nitrogen originated from the nitrates. Dehérain and Maquenne<sup>66</sup> demonstrated that nitrate decomposition in the soil takes place only in the absence of atmospheric oxygen and in the presence of an abundance of organic matter. The process is checked by heating the soil or treating it with chloroform, which results in the destruction of the bacteria responsible for the reduction of the nitrates.<sup>67</sup> In the decomposition of organic nitrogenous compounds, free from nitrates, both in the presence and absence of oxygen, nitrogen gas is not produced; when nitrates are present, an active reduction takes place in the absence of oxygen, with the formation of gaseous nitrogen and various oxides of nitrogen.<sup>68</sup> This reduction diminishes with an increase in the amount of oxygen present but does not stop entirely. Even those investigators who believed at first that denitrification is a purely chemical process, carried out by means of the soil colloids, were convinced by later studies that nitrate reduction is not of a chemical nature.<sup>69</sup>

Bréal<sup>70</sup> found that a nitrate solution to which straw or horse manure

<sup>63</sup> Gerlach and Vogel. Centrbl. Bakt. II, 7: 609-623. 1901.

<sup>64</sup> Davy, 1819 (p. 117).

<sup>65</sup> Dietzell, B. E. Ztschr. Landw. Ver. Bayern. 72: 186-201. 1882.

<sup>66</sup> Dehérain, P. P. and Maquenne. Compt. Rend. Acad. Sci. 95: 691-693, 732-734, 854-856. 1882.

<sup>67</sup> Ehrenberg, A. Ztschr. physiol. Chem. 11: 145-178, 438-471. 1886.

<sup>68</sup> Tacke, Br. Landw. Jahrb. 16: 917-939. 1888.

<sup>69</sup> Vogel, J. Centrbl. Bakt. II, 34: 540. 1912; Landw. Vers. Sta. 78: 265-301. 1912; 82: 159-160. 1913.

<sup>70</sup> Bréal, E. Ann. Agron. 18: 181-195. 1892. Compt. Rend. Acad. Sci. 114: 681-684. 1892.

is added liberates a great deal of gaseous nitrogen. Wagner<sup>71</sup> then attempted to draw, on insufficient ground, broad generalizations concerning the reduction of nitrates to gaseous nitrogen by denitrifying bacteria in manure, even when added to the soil.

Gayon and Dupetit isolated from the soil, in 1886, two bacteria (*B. denitrificans*  $\alpha$  and  $\beta$ ) capable of reducing nitrates to gaseous nitrogen. The two organisms were cultivated upon a medium having the following composition:<sup>72</sup>

1. Distilled water.....	250 cc.	2. Distilled water.....	500 cc.
KNO <sub>3</sub> .....	2 grams	Citric acid.....	5 grams
Asparagine.....	1 gram	KH <sub>2</sub> PO <sub>4</sub> .....	2 grams
		MgSO <sub>4</sub> .....	2 grams
		CaCl <sub>2</sub> .....	0.2 gram
		FeCl <sub>3</sub> .....	Trace

Solution 2 is neutralized with a 10 per cent solution of NaOH or KOH, with phenolphthalein as an indicator. The two solutions are mixed and made up to 1000 cc. with distilled water.

For the isolation of denitrifying organisms, various other media can be used: (1) 1000 cc. water, 10 grams glucose, 6 grams NaNO<sub>3</sub>, 6 grams NaCl, 0.02 gram Ca<sub>2</sub>(PO<sub>4</sub>)<sub>2</sub>. (2) 100 cc. water, 0.5 to 1.5 grams NaNO<sub>3</sub>, 20 to 50 grams glycerol, 7 grams malic acid (neutralized with sodium carbonate), 0.5 gram sodium phosphate, 0.5 gram NaCl, 0.5 gram Na<sub>2</sub>CO<sub>3</sub>, 0.1 gram MgSO<sub>4</sub>. (3) 1000 cc. water, 20 grams of calcium tartrate, citrate or malate, 10 to 20 grams KNO<sub>3</sub>, 0.5 gram K<sub>2</sub>HPO<sub>4</sub>.

In the absence of free atmospheric oxygen, practically all the nitrate nitrogen can be transformed into gaseous nitrogen. When asparagine is replaced by sugar, the ammonia otherwise produced from the asparagine is not formed. In the reduction of nitrate to free nitrogen (so-called "nitrate fermentation"), there is an abundant accumulation of alkali, till the process is stopped when the alkali concentration is equivalent to 1 per cent sodium carbonate.<sup>73</sup> When the alkali is neutralized by means of an acid, nitrate reduction continues further, until all the nitrate has disappeared.<sup>74</sup> The organisms are very sensitive to free acids. The optimum reaction for the reduction of nitrates is pH 7.0 to 8.2;

<sup>71</sup> Aeby, J., Dorsch, R., Matz, Fr. and Wagner, P. Landw. Vers. Sta. 48: 247-360. 1897.

<sup>72</sup> Giltay, E. and Aberson, G. Arch. Neerland. 25: 341. 1892; Ampola and Ulpiani. Gazz. chim. ital. 1898, 410; Maassen, 1901 (p. 154).

<sup>73</sup> Burri, R. and Stutzer, A. Centrbl. Bakt. II, 1: 257-265, 350-364, 392-398, 422-432. 1895; 2: 473-474. 1896.

<sup>74</sup> Zacharowa, T. M. Trans. Institute of Fertilizers, No. 15, 1923, Moskau.

the limiting reactions are pH 5.5 and pH 9.8. The optimum reaction for the reduction of nitrites is pH 5.5 to 7.0.

The reduction of nitrates to atmospheric nitrogen may be a result of associative action of two bacteria, one (*Bact. coli*) reducing the nitrate to nitrite and the other (*Bact. denitrificans* I) reducing the nitrite to atmospheric nitrogen.<sup>75</sup> In case of associative growth, the aerobic form removes the free oxygen, thus enabling the other organism to become the denitrifier. The four species found by Maassen capable of reducing nitrate to gaseous nitrogen were *Bact. fluorescens liquefaciens*, *Bact. fluorescens* from blood, *Bact. pyocyaneum* and *Bact. praepollens*. Other investigators<sup>75</sup> also found *Bact. pyocyaneum*, *Bact. hartlebii* and fluorescent bacteria among the most active denitrifying organisms.

Among the forms capable of reducing nitrates completely to gaseous nitrogen, we may include organisms isolated from horse manure, cattle excreta (*Bact. denitrificans agilis*)<sup>76</sup> and soil.<sup>77</sup> Van Iterson<sup>78</sup> demonstrated the presence in the soil of various bacteria, including *Bact. stutzeri*, *Bact. denitrofluorescens* and *Bact. vulpinus*, which reduce nitrates to gaseous nitrogen in the presence of small quantities of organic matter. In the same soil, where nitrification takes place under aerobic conditions, denitrification will take place in the absence of free oxygen.

The following authentic organisms capable of reducing nitrates to atmospheric nitrogen have been isolated and described (some of these are probably only varieties of other species which do not denitrify):

*Bact. denitrificans* (= *Bact. denitrificans* I Burri and Stutzer, *Pseud. stutzeri* Mig.) L and N (1.5 to 3 by 0.7 $\mu$ ), a motile, non-spore forming, aerobic organism.

*Bact. stutzeri* (= *Bact. denitrificans* II Burri and Stutzer, *Bact. nitrogenus* Mig.) L and N (2 to 4 by 0.7 to 0.8 $\mu$ ), a motile, non-spore forming, facultative anaerobic organism, isolated from straw and horse manure.<sup>79</sup>

*Bact. kunnemanni* (= *Bact. denitrificans* III Kunnemann), a motile, non-spore forming organism.

<sup>75</sup> Sewerin, S. A. Centrbl. Bakt. II, 3: 504-517, 554-563. 1897; 22: 348-370. 1909; 25: 479-492. 1909; Christensen, H. R. Centrbl. Bakt. II, 11: 190-194. 1904; Fred, E. B. Centrbl. Bakt. II, 32: 421-449. 1911.

<sup>76</sup> Schirokikh, J. Centrbl. Bakt. II, 2: 204-207. 1896; Ampola, G. and Garino, E. Centrbl. Bakt. II, 2: 670-676. 1896; 3: 309-310. 1897.

<sup>77</sup> Jensen, 1897-8 (p. 154); Höflich, C. Centrbl. Bakt. II, 8: 245-248, 273-278, 305-308, 336-339, 361-367, 398-406. 1902; Cingolani, M. Staz. Sper. Agr. ital. 41: 521-538. 1908; Ann. Staz. Chim. Agr. Spes. Roma (2), 2: 274. 1908. (Centrbl. Bakt. II, 23: 238. 1909.)

<sup>78</sup> van Iterson, C. Centrbl. Bakt. II, 12: 106-116. 1904.

<sup>79</sup> Kunnemann, O. Landw. Versuchsta. 50: 65-113. 1898.

*Bact. denitrificans agilis*<sup>80</sup> (1 to 1.5 by 0.1 to 0.3 $\mu$ ), a motile, peritrichic, non-spore forming organism; gram-negative, facultative anaerobic, granulated and developing slow; according to Löhnis this is a denitrifying variety of *Bact. radiobacter*.

*Bact. ulpiani* (= *Bac. denitrificans* VI Ampola et Ulpiani), a motile, non-spore forming, gram-negative organism.

*Vibrio denitrificans*<sup>81</sup> (2 to 4 by 0.5 $\mu$ ), a motile, non-spore forming organism.

*Bac. schirokikhi*, a motile, spore-forming, proteolytic, aerobic organism.

*Bact. praepollens*, a small, non-motile, obligate aerobic organism, reducing only nitrites.

*Bac. nitroxus* (No. 62, Pl. IX) comprising bacilli of variable dimensions, globous, pyriform, filiform; they take the form of Clostridia at the time of spore formation, giving an intense glycogen reaction; facultative anaerobic; on repeated transfer under aerobic conditions may lose faculty of reproduction; gelatin is liquefied.

In addition to these and the above mentioned bacteria, we may also call attention to a few other denitrifying forms which were isolated, such as *Bact. fulvum*, *Bact. hartlebii*, *Bact. centropunctatum*, *Bact. nitrovorum*, *B. porticensis*, etc. Most of these organisms are strict aerobes, some being capable of decomposing proteins actively. Most of them grow on nitrate (0.2 to 1.0 per cent) media, with the formation of a gas (largely N, some CO<sub>2</sub>) and nitrite. In the absence of free oxygen, these organisms can exist anaerobically in the presence of nitrate.

A thermophilic denitrifying bacillus (3.5 to 7 by 1 to 1.8 $\mu$ ), facultative anaerobic, reducing nitrates with the formation of gas and growing at high temperatures (52°C.) has also been described.<sup>82</sup>

Several organisms reducing nitrates are capable of obtaining their energy from inorganic compounds. *Thiob. denitrificans* oxidizes sulfur and reduces nitrates to nitrogen gas. This organism, or rather group of organisms, is widely distributed in the soil. Thiosulfate can be oxidized by the organism under anaerobic conditions only in the presence of nitrate as a source of oxygen. The utilization of the energy obtained by the oxidation of hydrogen gas for the reduction of nitrates has been pointed out by Niklewski<sup>83</sup> for *H. agilis*.

The decomposition of cellulose in the soil may be carried on by the

<sup>80</sup> Ampola and Garino, 1896-1897 (p. 158); Kuntze, W. Centrbl. Bakt. II, 13: 1-12. 1904.

<sup>81</sup> For a description of this and the following organisms, see Sewerin, 1897 (p. 158); Jensen, 1898 (p. 154); Maassen, 1899 (p. 154); Beijerinck, M. W. and Minkman, D. C. J. Centrbl. Bakt. II, 25: 30-63. 1910; Centrbl. Bakt. II, 23: 672-726. 1909.

<sup>82</sup> Ambroz, 1913 (p. 151). A detailed review of the morphology of the denitrifying bacteria is given by G. K. Burgwitz. Bull. Inst. Agr. Microb. U. S. S. R. 4: 213-255. 1930.

<sup>83</sup> Niklewski, 1914 (p. 97).



symbiotic action of two bacteria, one reducing nitrate to atmospheric nitrogen and the other decomposing the cellulose; the decomposition products of the cellulose are used by the nitrate reducing organism as a source of energy, which enables it to reduce the nitrate, while the oxygen thus liberated is utilized by the cellulose decomposing organism, under anaerobic condition.<sup>84</sup>

*Bacteria reducing sulfates to H<sub>2</sub>S.* Several heterotrophic bacteria are capable of producing hydrogen sulfide as a result of reduction of sulfates and other oxygen-rich sulfur compounds (like thiosulfates). *Microspira desulfuricans* (No. 63, Pl. IX) was first studied by Beijerinck and isolated in pure culture by Van Delden,<sup>85</sup> on the following medium:

K <sub>2</sub> HPO <sub>4</sub> .....	0.5 gram	MgSO <sub>4</sub> or CaSO <sub>4</sub> .....	1.0 gram
Sodium lactate.....	5.0 grams	FeSO <sub>4</sub> .....	Trace
Asparagine.....	1.0 gram	Tap water.....	1000 cc.

<sup>84</sup> Gerretsen, Meddel. Proef. Java Suiker. No. 3. 1921.

<sup>85</sup> Beijerinck, M. W. Centrbl. Bakt. II, 1: 1-9, 48-59, 104-114. 1895; Van Delden, A. Centrbl. Bakt. II, 11: 81-94, 113-118. 1904; Baars, J. K. Over sulfaatreductie door bacteriën. Delft. 1930.

## PLATE VII

### HETEROTROPHIC AEROBIC AND ANAEROBIC BACTERIA

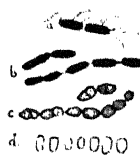
50. *Bac. mycoides*, × 660 (from Conn).
51. *Bac. cereus*, × 660 (from Conn).
52. *Bac. megatherium*, × 660 (from Conn).
53. *Bac. simplex*, × 660 (from Conn).
54. *Bact. vulgare*, × 660 (after Omeliansky).
55. *Bact. pyocyaneum*, × 660 (after Omeliansky).
56. *Bact. fluorescens*, × 660 (after de Rossi).
57. *Bac. butyricus*: *a*, non-spore forming; *b*, spore forming, × 660 (from Omeliansky).
58. *Bac. sporogenes*: *a*, 24 hour culture upon glucose bouillon; *b*, flagella, stained by Loeffler's method (from Weinberg and Seguin).
59. *Bac. putrificus*, 48 hour old colony in deep glucose agar (from Weinberg and Seguin).
60. *Bac. probatus*: *A*, non-sporulating bacilli of a fresh agar culture; *B*, sporulating bacilli of an agar culture 4-8 days old; *C*, spores with adhering membrane of a 2 to 3 week old culture upon potato, × 1300 (after Viehoveer and de Rossi).
61. *Sarcina ureae*, × 660 (after Omeliansky).
62. *Bac. nitrozeus*, 3-day old culture, grown at 30°, × 480 (after Beijerinck and Minkmann and de Rossi).
63. *Spirillum desulfuricans*, × 660 (after Beijerinck and Omeliansky).



50



51



52



53



54



55



56



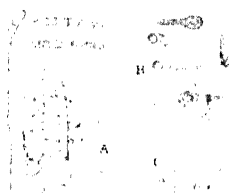
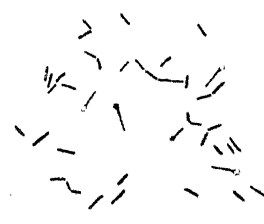
57



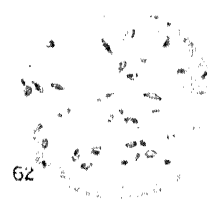
58



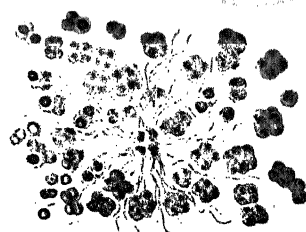
59



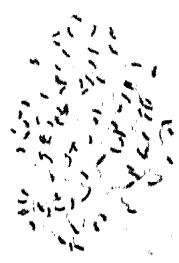
60



62



61



63

symbiotic action of two bacteria, one reducing nitrate to atmospheric nitrogen and the other decomposing the cellulose; the decomposition products of the cellulose are used by the nitrate reducing organism as a source of energy, which enables it to reduce the nitrate, while the oxygen thus liberated is utilized by the cellulose decomposing organism, under anaerobic condition.<sup>84</sup>

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<sup>84</sup> Gerretsen, Meddel. Proef. Java Suiker. No. 3. 1921.

<sup>85</sup> Beijerinck, M. W. Centrbl. Bakt. II, 1: 1-9, 48-59, 104-114. 1895; Van Delden, A. Centrbl. Bakt. II, 11: 81-94, 113-118. 1904; Baars, J. K. Over sulfaatreductie door bacteriën. Delft. 1930.

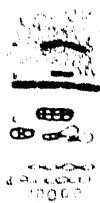
## PLATE VII

### HETEROTROPHIC AEROBIC AND ANAEROBIC BACTERIA

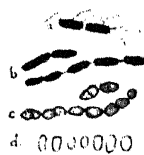
50. *Bac. mycoides*, × 660 (from Conn).
51. *Bac. cereus*, × 660 (from Conn).
52. *Bac. megatherium*, × 660 (from Conn).
53. *Bac. simplex*, × 660 (from Conn).
54. *Bact. vulgare*, × 660 (after Omeliansky).
55. *Bact. pyocyaneum*, × 660 (after Omeliansky).
56. *Bact. fluorescens*, × 660 (after de Rossi).
57. *Bac. butyricus*: *a*, non-spore forming; *b*, spore forming, × 660 (from Omeliansky).
58. *Bac. sporogenes*: *a*, 24 hour culture upon glucose bouillon; *b*, flagella, stained by Loeffler's method (from Weinberg and Seguin).
59. *Bac. putrificus*, 48 hour old colony in deep glucose agar (from Weinberg and Seguin).
60. *Bac. probatus*: *A*, non-sporulating bacilli of a fresh agar culture; *B*, sporulating bacilli of an agar culture 4-8 days old; *C*, spores with adhering membrane of a 2 to 3 week old culture upon potato, × 1300 (after Viehoveer and de Rossi).
61. *Sarcina ureae*, × 660 (after Omeliansky).
62. *Bac. nitrozus*, 3-day old culture, grown at 30°, × 480 (after Beijerinck and Minkmann and de Rossi).
63. *Spirillum desulfuricans*, × 660 (after Beijerinck and Omeliansky).



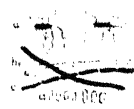
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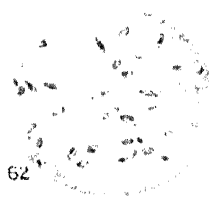
58



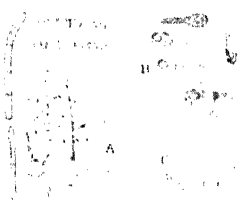
59



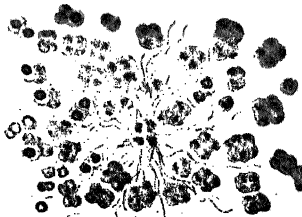
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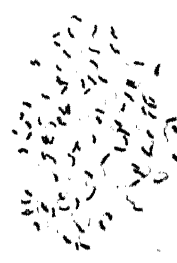
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62



63





The medium is filled to the neck of the bottles, then inoculated and incubated at 25°C. Sulfur reduction becomes evident by a change in color due to formation of  $H_2S$ . The bacterium can be isolated from the soil when some sodium sulfite is added to the medium. The presence of organic substances as sources of energy and anaerobic conditions are required for the action of the organism. For the isolation of pure cultures, 10 per cent gelatin or 2 per cent agar is added to the above medium; in place of  $FeSO_4$  a trace of  $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$  together with some sodium carbonate is used. In 3 to 6 days small black colonies appear. Sulfur is deposited in the colony, on the solid medium among the bacterial cells, due to the incomplete reduction of the sulfate. The organism is a very motile spirillum, 4 by  $1\mu$  in size and is strictly anaerobic. Another organism (*Microspira aestuarii*) was isolated from sea water. Certain thermophilic bacteria (*Vibrio thermo desulfuricans*) are capable of reducing sulfates.<sup>86</sup>

The three forms of sulfate-reducing bacteria are closely related to one another and have the ability, apart from all other bacteria, to utilize sulfates and thiosulfates as sources of oxygen in the absence of free atmospheric oxygen.

*Organisms decomposing urea.* Pasteur<sup>87</sup> was the first to recognize in 1860 that ammonia formation from urea is brought about by a living organism, namely *Torula ammoniacale*. It was later found that organisms capable of decomposing urea are found in most families of bacteria, actinomyces and fungi, but only certain specific bacteria, whose metabolism is closely connected with the transformation of this substance, are termed urea bacteria. These are divided into cocci and bacilli. The former are usually destroyed at 60° to 70°, while the latter, due to their ability to form endospores, can withstand heating at 90° to 95° for several hours. The optimum temperature for the action of these organisms is about 30°C. They usually thrive best in media containing urea (2 per cent), particularly when made alkaline with ammonium carbonate (2 to 3 grams per liter). The accumulation of ammonium carbonate from the hydrolysis of the urea is so great, in many instances, as to kill the organisms themselves. A rapid urea decomposition does not necessarily accompany a rapid growth. The urea bacteria differ in their oxygen tension; most of them are aerobic, although the amount of oxygen required may be rather small.

The urea splitting bacteria are commonly found in great abundance

<sup>86</sup> Elion, L. Centrbl. Bakt. II, 63: 58-67. 1924.

<sup>87</sup> Pasteur, L. Compt. Rend. Acad. Sci. 50: 849-854. 1860.

in soil, manure, dust and water. Miquel<sup>88</sup> found urea organisms in the canal waters of Paris, fifty-two forms in the sewage waters and sixty-six in the drainage of the privy closets. The urea bacteria of the surface soil form 1 to 2 per cent of the total number of the organisms developing on the plate. Manure and urine contain 10 per cent of their flora as urea bacteria. Frequently the urea is so rapidly decomposed by the bacteria as to lead to actual losses of ammonia. This can be prevented however, by a proper mixture of the urea with the soil.<sup>89</sup> Certain soils, however, possess only a feeble power of transformation of urea into ammonium carbonate. The addition of fresh manure or of soy bean urease favors this process and as a result of that, the rapidity of nitrogen utilization by plants. The active bacteria seem to diminish in the soil in course of time.<sup>90</sup>

The isolation and cultivation of the urea bacteria does not present any great difficulty. The selective and enrichment cultivation<sup>91</sup> can be readily utilized for this purpose.

For the isolation of the organisms, the following two solutions can be used:

1. Tap water.....	1000 cc.	2. Tap water.....	1000 cc.
$K_2HPO_4$ .....	0.5 gram	$K_2HPO_4$ .....	0.5 gram
Calcium citrate or		Ammonium	
tartrate.....	10.0 grams	malate.....	10.0 grams
Urea.....	30.0 grams	Urea.....	50.0 grams

One hundred cubic centimeters of either solution is inoculated with 2 grams of soil and incubated at 23° or 33°; in 36 to 48 hours, the medium becomes well inoculated with urea bacteria. After two or three transfers, the organisms are readily obtained in pure culture. A medium consisting of 50 grams of urea, 0.5 gram  $K_2HPO_4$ , 100 cc. of soil extract and 900 cc. of tap water may be used. Peptone gelatin containing 2 to 5 per cent of urea forms a good solid medium. A few days after inoculation, often only after twenty-four hours, most of the visible colonies will be found surrounded with a halo. This is composed of dumb-bell shaped crystals insoluble in water and consisting of carbonate and phosphate of calcium which were precipitated from the medium as a result of the formation of ammonium carbonate from the urea. The stronger the action of the bacteria, the wider is the zone. The halo of crystals either surrounds the colony to a width of several millimeters or rapidly covers, in 24 hours, the whole plate. The urea organisms are thus readily recognized and are then transferred upon the specific media.

<sup>88</sup> Miquel, P. Lafar's Handb. techn. Mykol. 3: 71-85. 1904.

<sup>89</sup> Littauer, F. Ztschr. Pflanzenernähr. Düng. 3A: 65. 1925.

<sup>90</sup> Bordas, J. and Mathieu, G. Ann. Sci. Agron. 46: 561-574. 1929.

<sup>91</sup> Beijerinck, M. W. Centrbl. Bakt. II, 7: 33-60. 1901; Duggeli. Naturw. Wochschr. 14: 305. 1915; Söhngen, N. L. Centrbl. Bakt. II, 23: 91-98. 1909; Löhnis, 1905 (p. 13).

Ordinary bouillon, with or without peptone, yeast water, peptone solution, which have been made alkaline and to which 0.1 to 0.2 per cent of urea has been added are quite suitable for the cultivation of the organisms. Viehovever<sup>92</sup> used, for the isolation of *Bac. probatus* and other urea bacteria, a medium consisting of:

Water.....	1000 cc.	NaCl.....	0.1 gram
K <sub>2</sub> HPO <sub>4</sub> .....	1 gram	FeCl <sub>3</sub> .....	0.01 gram
CaCl <sub>2</sub> .....	0.1 gram	Urea.....	20.0 grams
MgSO <sub>4</sub> .....	0.3 gram	Liebig's beef extract.	5.0 grams

Agar is added to this solution when a solid medium is wanted for the isolation of the bacteria from colonies. Ammonium carbonate or urea agar are prepared by adding 3 grams of the first or 3 to 30 grams of urea to a medium consisting of 1500 parts of water + 30 agar + 6 peptone + 4 Liebig's beef extract + 1 NaCl + 5 glucose.

*Classification and description of urea bacteria.* The urea bacteria include both anaerobic and aerobic forms. They can be divided into four groups:

I. Spore-bearing cocci. *Planosarcina ureae* Beij. forms packets on solid and liquid media, of 4 to 8 cells (0.7 to 1.2 $\mu$ ), and is motile by means of long flagella. The spherical endospores (0.6 $\mu$ ) can withstand 80°C. for ten minutes.

II. Non-spore bearing cocci. *Urococcus van tieghemi* Miquel (Syn. *Torula ammoniacale* Pasteur, *Mic. ureae* Cohn), 1 to 1.5 $\mu$  in diameter, occurring in twos, often in chains; *Mic. ureae liquefaciens* Flügge, *Uros. hansenii* Miquel and a number of other *Urosarcinae* and *Micrococci* described by Miquel, Rochaix and Dufourt<sup>93</sup> as well as the common species *Mic. pyogenes* and *Strept. pyogenes*.

III. Spore-bearing bacilli. These include the *Urobacillus pasteurii* (Miquel) Beij., 1 to 1.2 by 2.5 $\mu$ , single or short chains, motile, forming egg-shaped endospores, persisting in dry soil for many years, decomposing urea very actively; *Urob. duclauxii*, *Urob. freudenreichii*, *Urob. maddoxii* and other species described by Miquel; *Urob. leubii* Beij., *Bac. probatus* Vieh., *Bac. ureae* II and III Burri. According to Löhnis, *Bac. mycoides* and *Bac. megatherium*, which decompose urea, belong here, as well as other spore-bearing bacteria (so-called putrefactive forms), such as the anaerobic *Bac. putrificus*, and *Bac. perfringens*.

IV. Non-spore bearing bacteria. *Bact. ureae* Leube, *Bact. ureae* I Burri, *Urobact. schutzenbergii* Miquel, *Urobact. miquelii* Beij., *Urobact. jackshii* Söhnngen, *Urobact. beijerinckii* Christ., as well as a number of

<sup>92</sup> Viehovever, A. B. Centrbl. Bakt. 39: 209-359. 1913.

<sup>93</sup> Rochaix and Dufourt. Compt. Rend. Soc. Biol. 69: 312-314. 1910.



common bacteria like *Bact. coli*, *Bact. prodigiosum*, *Bact. fluorescens*, *Bact. vulgare*, etc.

According to Löhnis,<sup>94</sup> the urea-decomposing power of the various urea bacteria quickly ceases when these organisms are kept in culture. They cannot, therefore, be considered as representatives of separate genera, but merely as varieties of common species, like *Bact. vulgare*, *Bact. coli*, *Bact. prodigiosum*, *Bact. fluorescens*, *Bact. erythrogenes*. Most of the *Urococcus* species, which are distinguished only by degree of pigment formation and gelatin liquefaction, belong chiefly to the *Microc. ureae* Cohn. *Bact. ureae* Leube, named by Miquel *Urobacillus leubii*, belongs to the *Bact. vulgare* group; the same is true of *Urob. miquelii* Beij. and other *Urobacilli* (like *Urob. jakschii* Söhngen). This assumption is justified in view of the fact that a number of common soil bacteria, including various cocci, non-spore-forming and spore-forming bacteria, are capable of decomposing urea.

Söhngen<sup>95</sup> described two new non-spore-forming bacteria decomposing urea in the absence of proteins. An aerobic non-spore-forming, rod-shaped organism (*Urob. beijerinckii*), 1.25 by 0.75 to 1 $\mu$  in size, is capable of using urea both as a source of carbon and nitrogen, in the complete absence of other organic substances.<sup>96</sup> Glucose cannot be utilized and may even injure the urea-splitting power of the organism. Humic acid was found to have a favorable influence upon the decomposition of urea.

Viehoever found that most of the common urea bacteria, such as *Urobacillus pasteurii*, *Urob. leubii* and probably also *Urob. maddoxii*, *Bac. ureae* II and III, *Bact. ureae* could be combined into one species, *Bac. probatus* (No. 60, Pl. VII).

This organism was obtained by heating some soil at 100°C., then inoculating into a medium containing 1 gram  $K_2HPO_4$ , 0.1 gram  $CaCl_2$ , 0.3 gram  $MgSO_4$ , 0.1 gram  $NaCl$ , 0.01 gram  $FeCl_3$ , 20 grams urea and 5 grams Liebig's beef extract per liter. On incubating for three weeks at 28°, the culture was found to contain rod-shaped organisms 10 $\mu$  long which formed spores 1 $\mu$  in size. A small amount of the culture was boiled for one minute at 100° and the organism was obtained in pure culture by the dilution method, using the above medium with the addition of agar. The maximum acid tolerance was found to be two drops  $N H_2SO_4$  per 5 cc. of agar medium ( $\frac{1}{3}$  concentration of nutrients). The maximum alkalinity was expressed by 2 per cent dehydrated  $Na_2CO_3$  (optimum 0.2 per cent) or 22 to 25 per cent of ammonium carbonate (optimum 0.3 per cent). The maximum

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<sup>94</sup> Löhnis and Kuntze, 1908 (p. 28).

<sup>95</sup> Söhngen, 1909 (p. 162).

<sup>96</sup> Christensen, H. R. Centrbl. Bakt. II, 27: 337-362. 1910.

concentration of urea tolerated by the organism was 30 to 40 per cent, the optimum was 3 per cent.

*Bacillus probatus* A. M. et Viehoveer is motile, with peritrichic flagella and rounded ends; it forms on ammonium carbonate or urea agar chains of 2 to 3 cells. The cells vary greatly in size, usually 1 by 0.4 to 0.8 $\mu$ , reaching on some media a size of 3 to 10 by 0.7 $\mu$ . The organism is gram-positive, spore-forming, aerobic. The size of the spores on the ammonium carbonate agar ranges from 0.5 to 1 by 0.6 to 1.2 $\mu$  to almost spherical, 0.8 to 0.9 $\mu$  in diameter. The spores appear in 3 to 4 days on the ammonium carbonate agar at 28°C., more or less at the end of the cell, as drum-shaped or spindle-shaped, swollen sporangia (Pl. IX). Development is weak on common nutrient and nutrient-glucose agar. White to grayish-opalescent colonies are formed in three days on the carbonate or urea agar. In peptone broth containing 0.2 per cent ammonium carbonate, indol, H<sub>2</sub>S and trimethylamine are formed. No gas is formed from sugars, acid only from glucose. Ammonia is formed from nitrites. Crystals of calcium carbonate and phosphate are formed on urea agar. The enzymes catalase, urease, reductase are formed, but not oxidase or amylase. The minimum temperature for growth is 3° to 5°, optimum 28° to 35°, maximum for spore-formation 42° to 43°, for spore germination 45° to 47°, for growth 44° to 45°. Minimum oxygen tension per liter for spore germination and growth is 4 mgm., for spore formation 10 mgm. Maximum oxygen tension for spore germination is about 10 atmospheres, for growth 5 atmospheres, and for spore formation 1 to 5 atmospheres. The spores are killed in 11.5 to 12.5 minutes at 100°C., in 9 to 10 hours at 80°C.

Geilinger<sup>97</sup> made a detailed study of the biology of urea-decomposing bacteria, with a view of preventing the rapid loss of nitrogen due to the decomposition of urea in the manure pile. Only 5.6 per cent of the urea organisms isolated from soil and manure were able to live and decompose urea in the absence of oxygen. Some organisms were found to be obligate anaerobes and were able to thrive in the presence of a mere trace of residual oxygen. A series of bacteria capable of decomposing urea at low temperatures, even below 0°C., were isolated from water and curative muds.<sup>98</sup>

The urea bacteria have a very low acid limit, which is, in the case of *Urobacillus duclauxii* pH 6.6, for *Ur. maddoxii* pH 7.0 and for *Ur. pasteurii* pH 8.1. The optimum reaction for the growth of the first two organisms is pH 7.4-7.7 and pH 8.2 respectively. The favorable effect of peat litter in preventing losses of ammonia from manure was believed to be due to the checking of the growth of the urea bacteria through the high acidity of the peat.<sup>99</sup>

*Bacteria decomposing uric and hippuric acids.* The soil harbors a

<sup>97</sup> Geilinger, H. Centrbl. Bakt. II, 47: 245-301. 1917.

<sup>98</sup> Rubentschik, L. Centrbl. Bakt. II, 64: 116-174. 1925; 66: 161-180. 1926.

<sup>99</sup> Kusnetzow, S. J. Trans. Sci. Inst. Fertil. (Russian), 76: 116-122. 1930.

number of bacteria capable of obtaining their carbon and nitrogen needs from uric and hippuric acids. By the use of a medium containing 0.3 per cent uric acid and 0.05 per cent  $K_2HPO_4$ , various bacteria belonging to the *Bact. radiobacter*, *Bact. fluorescens*, *Bact. pyocyaneum* and other groups can be isolated from the soil. *Aerobacter aerogenes* was isolated<sup>100</sup> on a medium consisting of 5 grams  $NaCl$ , 0.2 gram  $MgSO_4$ , 0.1 gram  $CaCl_2$ , 1.0 gram  $K_2HPO_4$ , 30 grams glycerol and 0.2 gram of uric acid in 1000 cc. of distilled water.

A medium containing 1 per cent sodium hippurate, 0.2 per cent  $K_2HPO_4$ , and 0.1 per cent  $MgSO_4$  was used to demonstrate the presence in soil of microorganisms capable of transforming hippuric acid; these organisms are found in the surface layer of the soil in larger numbers and are more active than in the subsoil.<sup>101</sup> Thirty-four species of bacteria were isolated<sup>102</sup> from soil, manure, urine, etc.; of these, twenty-eight decomposed hippuric acid and the others decomposed urea and uric acid. All those bacteria that decomposed urea also decomposed uric acid and vice versa; but those that decomposed hippuric acid did not necessarily decompose urea and uric acid, and vice versa.

Stapp<sup>103</sup> employed, for the isolation of uric and hippuric acid bacteria, the following two media:

I. Uric acid.....	0.5 gram	II $KH_2PO_4$ .....	0.50 gram
$Na_2HPO_4$ .....	3.0 grams	$MgSO_4$ .....	0.25 gram
Mineral solution....	50 cc.	Sodium hippurate	1.25 grams
Water.....	450 cc.	Water.....	500 cc.

The mineral solution used in Medium I was that of A. Meyer:

$KH_2PO_4$ .....	1.0 gram	$NaCl$ .....	0.1 gram
$CaCl_2$ .....	0.1 gram	$Fe_2Cl_6$ .....	0.01 gram
$MgSO_4 \cdot 7H_2O$ .....	0.3 gram	$H_2O$ .....	1000 cc.

Portions of the media (50 cc.) were placed into 200 cc. Erlenmeyer flasks and inoculated with soil or feces of various animals.

Six species of bacilli were carefully described.

1. *Bac. cobayae* A. M. & S. a non-motile, spore-forming organism. The cells attain a size up to  $5.5\mu$  long (usually  $4\mu$ ) and 1 to  $1.2\mu$  in diameter. The ellipsoidal to cylindrical spores, usually with convex poles, are  $1.4$  by  $0.8\mu$ . The organism forms diastase, protease, also  $H_2S$ , tryptophane and skatol; nitrates are reduced.

<sup>100</sup> Bierema, 1909 (p. 426); Morris, J. L. and Ecker, E. F. Jour. Inf. Dis. **34**: 592-598. 1924.

<sup>101</sup> Yoshimura, K. Bull. Coll. Agr. Tokyo. Imp. Univ. **2**: 221-223. 1895.

<sup>102</sup> Schnellmann, H. Diss. Göttingen. 1912.

<sup>103</sup> Stapp, C. Centrbl. Bakt. II, **51**: 1-71. 1920.

2. *Bac. capri* A. M. & S. is without flagella, up to  $6.2\mu$  long and 1 to  $1.1\mu$  in diameter; ellipsoidal or egg shaped spores are 1.4 by  $0.8\mu$ ; reduces nitrates and forms diastase.

3. *Bac. guano* A. M. & S. is a motile rod, with peritrichic flagellation, up to  $5.4\mu$  long and  $0.7\mu$  in diameter (2.8 to 3.4 by 0.6 to  $0.7\mu$ ); the ellipsoidal spores are 1.4 by  $0.8\mu$ ; weak reducing power, no diastase formation; gelatin is liquefied.

4. *Bac. musculi* A. M. & S., with peritrichic flagellation, is 4.5 to 5 by 1 to  $1.2\mu$ ; spores 1 to 2.2 by 0.6 to  $1.2\mu$  (1.8 by  $0.8\mu$ ); weak reducing power; diastase is formed; gelatin is liquefied.

5. *Bac. hollandicus* A. M. & S., with peritrichic flagellation, is 6 by 0.7 to  $0.8\mu$ ; spores  $1.6 \times 0.8\mu$ ; weak reducing power; diastase not formed, gelatin is liquefied.

6. *Bac. carotarum* Koch.

Ulpiani and Cingolani<sup>104</sup> isolated from pigeon manure a bacterium capable of decomposing guanin and guanidin, but not uric acid.

The chemical processes involved in the transformation of urea, uric and hippuric acids are discussed elsewhere.

<sup>104</sup> Ulpiani, C., and Cingolani, M. Atti R. Accad. Linc. Rend. Cl. Sci. fis. Mat. et Nat. (5), 14, pt. 2: 596-600. 1905.

## CHAPTER VII

### ANAEROBIC BACTERIA

*Oxygen tension in the growth of bacteria.* Pasteur<sup>1</sup> was the first to demonstrate that there are organisms, among them yeasts, which can live in the presence of only small traces of oxygen. Since the growth of the microorganisms is so abundant that the small amount of oxygen present is rapidly used up, it can be assumed that the greater part of their development takes place in the absence of free oxygen. Pasteur has further shown that, in the case of yeasts, growth in the absence of oxygen takes place only in the presence of utilizable sugar. Those organisms which are able to grow both in the presence and absence of free oxygen were termed by Liborius<sup>2</sup> "facultative anaerobes." It has also been observed by Pasteur that certain butyric acid bacteria grow abundantly in the liquid medium, through which a current of carbon dioxide is passed, but are destroyed, when a current of air is passed for 2 hours through the liquid. Those organisms, which are unable to thrive under partial oxygen pressure and cannot withstand even small amounts of oxygen, were termed by Liborius "obligate anaerobes." Beijerinck<sup>3</sup> divided the bacteria into two groups, according to their oxygen need: (1) "aerophile," or those requiring a high oxygen tension, including the aerobes and facultative anaerobes, which can grow in ordinary atmosphere; and (2) "microaerophile," or those organisms that require a more or less low oxygen tension and do not grow readily in ordinary atmosphere. The influence of oxygen on some bacteria was illustrated by the accumulation of the cells in a hanging drop preparation; the aerophiles gathered in the outer zone, while the microaerophiles massed together where the oxygen tension was least. The spirillum type was intermediate. Burri<sup>4</sup> could not agree with this division and suggested that the terminology of Liborius is much more

<sup>1</sup> Pasteur, L. Compt. Rend. Acad. Sci. 52: 360, 1260. 1861; Ibid., 56: 416, 1189. 1863; 75: 784. 1872; 80: 1875.

<sup>2</sup> Liborius, P. Ztschr. Hyg. 1: 115. 1886.

<sup>3</sup> Beijerinck, M. W. Centrbl. Bakt. 14: 827-845. 1893; Arch. Neerland, Ser. II, 2: 397. 1899; 9: 131. 1904.

<sup>4</sup> Burri, R. Centrbl. Bakt. II, 17: 804. 1907.

appropriate. Not only obligate anaerobic bacteria, but also the facultative forms were able to live in the complete absence of oxygen for a number of generations without being injured.

No general minimum oxygen tension could be found for all obligate anaerobic bacteria, but the various anaerobic forms varied in the limit of this tension:<sup>5</sup> the oxygen limit for the blackleg bacillus (*Bac. charuoei*) is 1.04 per cent oxygen in the atmosphere, 0.65 per cent for *Bac. tetani*, 0.27 per cent for *Clostridium butyricum* and 0.13 per cent for *Bactridium butyricum*; the obligate anaerobic bacteria could be so adapted as to withstand some amounts of oxygen. A typical obligate anaerobe has no minimum oxygen tension limit, it is characterized by the existence of a very low maximum oxygen tension and it can grow in the total absence of oxygen. We do not know of any true anaerobes which grow only in the complete absence of oxygen. Small quantities of free oxygen will even act as stimuli to obligate anaerobes.

The oxygen need of an organism was characterized<sup>6</sup> by the "cardinal points" for growth and spore formation, namely: minimum, optimum, and maximum, so that there is a gradual transition between aerobes and anaerobes. The following cardinal points for spore formation characterize a series of typical bacteria, atmospheric air at 18° and 750 mm. pressure containing 276 mgm. of oxygen per liter:

	MINIMUM	OPTIMUM	MAXIMUM
	mgm.	mgm.	mgm.
<i>Bac. amylobacter</i> .....	0	10 (?)	About 25
<i>Bac. asteroides</i> .....	0	100	5,600
<i>Bac. fusiformis</i> .....	6.8	70	1,061
<i>Bac. mycoides</i> .....	4.3	70	1,336
<i>Bac. simplex</i> .....	6.8	276	1,263
<i>Bac. subtilis</i> .....	4.3	400	4,317
<i>Bac. lactis</i> .....	20.0	400	1,336

A high maximum does not necessarily correspond to a high minimum. The first generation of anaerobes is more sensitive to oxygen than the following generations, which may even thrive better in the presence of a

<sup>5</sup> Chudiakow, N. Moskau. 1896 (Centrbl. Bakt. II, 4: 389-394. 1898).

<sup>6</sup> Meyer, A. Centrbl. Bakt., II, 15: 337. 1906. Centrbl. Bakt. I, 49: 305-316. 1909; II, 16: 386, 481-488, 577-588, 673-687. 1906. Wund, M. Centrbl. Bakt. 42: 97-101, 193-202, 289-296, 385-393. 1906.

limited oxygen supply than in its complete absence.<sup>7</sup> This points to adaptation in course of time. Even in the case of a single generation, the organism can withstand greater concentrations of oxygen after the growth of the culture has somewhat advanced than in the beginning. It has been claimed<sup>8</sup> that the growth of even obligate anaerobic bacteria is greatly injured in the complete absence of oxygen; however, Kürsteiner<sup>7</sup> demonstrated that both obligate and facultative anaerobes will thrive well for a number of generations in atmospheres free from oxygen. Free oxygen exerts an injurious effect upon obligate anaerobic bacteria, as pointed out already by Pasteur, the degree of injury depending on temperature, age and abundance of cells. In the following pages, the term "anaerobe" will be applied only to the so-called "obligate anaerobes." The presence of suspended particles, especially in case of colloidal suspensions, favors the growth of anaerobic bacteria possibly through their oxygen absorption.<sup>9</sup>

The more recent studies on oxidation-reduction processes in the growth of microorganisms have brought out the fact that only those bacteria are capable of growing anaerobically, which are capable of activating some constituent of the medium as a hydrogen acceptor. Some bacteria, like *B. vulgaris*, can activate nitrate and can, therefore, grow anaerobically in the presence of nitrate and certain hydrogen donors; *Bact. coli* and *Bact. prodigiosum* can activate nitrate, fumarate, malate and aspartate and can grow anaerobically, in the presence of any of these substances, and with glycerol as a hydrogen donor.<sup>10</sup> Recent important contributions point to the lack of catalase formation by anaerobic bacteria.<sup>11</sup> Peroxides are formed in the aerobic growth of bacteria and these peroxides would become injurious to the organisms if not for the catalase which is formed and which rapidly breaks up the peroxide into inactive oxygen and water. The anaerobic bacteria are unable to form catalase and are subject to the destructive action of the peroxide when grown under aerobic conditions. A number of

<sup>7</sup> Burri, 1907 (p. 168); Kürsteiner, J. Centrbl. Bakt. II, 19: 1-26, 97-115, 202-220, 385-399. 1907; Burri, R. and Kürsteiner, J. Ibid. 21: 289-307. 1908; Landw. Jahrb. d. Schweiz. 1909, 422.

<sup>8</sup> Fermi, C. and Bassu, E. Centrbl. Bakt. I, 35: 563-568, 714-722. 1905; 38: 138-145, 241-248, 369-380. 1905.

<sup>9</sup> Hata, S. Centrbl. Bakt. I, 46: 539-554. 1908; v. Lennep, R. Folia Microb. 1: No. 3. 1913.

<sup>10</sup> Quastel, 1925 (p. 410).

<sup>11</sup> McLeod, J. M. and Gordon, J. Jour. Path. Bact. 26: 326-331, 332-343. 1923; 28: 155-164, 147-153. 1925.

indicators are employed for measuring anaerobiosis or for determining the end point of free oxygen.<sup>12</sup>

For the existence of even obligate anaerobes in the soil we need not imagine a soil atmosphere free from atmospheric oxygen, but simply that anaerobic conditions, favorable for the activities of these bacteria, are produced due to the active utilization of the oxygen and production of CO<sub>2</sub> by aerobic organisms, which results in a reduction of the oxygen tension. This can be imitated artificially in the laboratory, when anaerobes are grown readily under ordinary conditions, in the presence of rapidly growing aerobic bacteria, like *Bac. subtilis*. Another illustration of this phenomenon is the growth of the two nitrogen-fixing organisms, the anaerobic *Cl. pastorianum* and the aerobic, rapidly growing *Azotobacter*. Exposure to oxygen has an injurious effect upon anaerobic organisms; the effect of air exposure upon the vegetative cells sets in only after 40 minutes, while the spores are not injured even after 3 hour exposure.<sup>13</sup>

*Methods of isolation of anaerobic bacteria from the soil.* There are a number of methods available for the isolation of anaerobic bacteria.<sup>14</sup> These have to be separated not only from aerobic organisms, but often also from other facultative or obligate anaerobic bacteria. The anaerobic, just as the aerobic, bacteria vary greatly in their food requirements and manner of growth, and the methods of isolation have to be adapted to the particular organism in question. There is a large number of species of anaerobes in the soil and it is insufficient to depend on microscopic examinations alone for demonstrating the existence of different forms. In all cases, the isolation and demonstration of the different species must be undertaken. In case the nature of an organism that is looked for is known, the development of a proper culture medium is simplified. An enriched culture is first prepared either by adding some soil to a specific medium kept under specific conditions, or the specific substance is added to the soil itself. An attempt is then made to obtain a culture of the specific bacterium free from accom-

<sup>12</sup> Hall, I. C. Jour. Bact. 6: 1-42. 1921. Kadisch, E. Centrbl. Bakt. I, Orig. 90: 462-468. 1923. Clark, W. M., Cohen, B. and Gibbs, H. D. U. S. Publ. Health Serv. Publ. Health Repts. Repr. No. 1017. 1925; von Riensdijk, M. Centrbl. Bakt. I, 88: 229-252. 1927.

<sup>13</sup> Bachmann, F. Centrbl. Bakt. II, 36: 1-41. 1912; Dorner, 1924 (p. 172).

<sup>14</sup> Heller, H. H. Jour. Bact. 6: 445-470. 1921. A complete review of the subject is given by M. Weinberg and B. Ginsbourg. Données récentes sur les microbes anaérobies et leur rôle en Pathologie. Masson et Cie. Paris. 1927.



panying non-spore-forming and spore-forming aerobic and anaerobic organisms.

For the separation of spore-forming organisms from non-spore-formers, whether aerobes or anaerobes, the soil is heated at 75° to 80°C., by placing 2 grams of soil in 10 cc. of sterile water and keeping in a water bath for 10 minutes. This leads to the destruction of all the vegetative cells, while bacterial spores are not injured. The soil is then inoculated into a proper medium, favorable for the development of the specific organism, which will develop under proper cultural conditions; the culture is then transferred repeatedly upon the selective medium and grown under strict anaerobic conditions. To purify anaerobes from aerobes, the method of Dorner<sup>15</sup> can be used. The deep agar tube, inoculated with the organisms, is allowed to cool and the agar to solidify. Two cubic centimeters of melted agar containing 0.2 per cent of mercury bichloride is then poured on the surface of the cooled agar and the tubes are closed with rubber stoppers. The aerobes are thus completely eliminated. However, neither of these methods will separate the facultative anaerobes from the obligate anaerobes.

To separate anaerobes from spore-forming aerobes, use is made of three procedures: (1) Strict anaerobic methods of cultivation. (2) The inhibitive action of gentian-violet on aerobic growth;<sup>16</sup> a 1:100,000 to 1:400,000 dilution of the dye in the agar medium is sufficient to render cultures of anaerobic bacteria free from spore-forming aerobes. (3) Anaerobic organisms are less sensitive than aerobes to pyrocatechin, chinon, sodium formate, and sodium sulphindigotate.<sup>17</sup>

The most difficult process, often involving a complicated technic, is the separation of spore-forming anaerobes from other spore-forming anaerobes. The improper separation has led to exaggerated claims for the nature and activities of the organisms. All or some of the following procedures are utilized for this separation:

1. Heating the soil so as to kill the vegetative forms, then introducing various dilutions of the heated soil suspension into the proper medium and making transfers from the culture at different stages of development (heating the culture every time a new transfer is made). The various spore-forming anaerobes sporulate at different periods of their development.

<sup>15</sup> Dorner, W. Landw. Jahrb. Schweiz. 1924, 1-28.

<sup>16</sup> Churchman. Jour. Exper. Med. 16: 2, 221, 1912; Hall, I. C. Jour. Inf. Dis. 27: 576-590. 1920.

<sup>17</sup> Kitasato, S., and Weyl, Th. Zeitschr. Hyg. 8: 41, 404. 1890; Rivas, D. Centrbl. Bakt. 32: 831-842. 1902.

2. Use of selective media stimulating the predominant development of the organism sought. This method has been of great help in the isolation of some important soil anaerobes. It is sufficient to mention that by the use of selective media and proper environmental conditions, such organisms as the anaerobic nitrogen-fixing forms, thermophilic and cellulose-decomposing forms and others were isolated. The specific medium is inoculated with an infusion of soil or manure, which may be previously heated, if the organism in question forms spores, and incubated at the desired temperature. The adjustment of the medium to specific reactions may often be sufficient to separate one group of bacteria from another, often even anaerobic forms from one another. For instance, the adjustment of the nitrogen-free glucose media to a pH of 5.5 will not only favor the development of the nitrogen-fixing *Clostridium pastorianum*, but will also prevent the development of the proteolytic bacteria, which usually accompany it.<sup>18</sup> For the enrichment of cellulose decomposing anaerobic bacteria, the use of a specific liquid medium or of a silica gel plate with cellulose as the only source of energy is recommended (p. 191). For the decomposition of hemicellulose, physiological salt solution containing cubes of potato has been used, while, for starch splitting organisms, media containing 1 per cent peptone broth and 5 per cent starch have been suggested.<sup>19</sup>

3. The addition of aniline dyes for the elimination of certain species of organisms.

4. Selective temperatures for the enrichment of various bacteria, developing preferably at the different temperatures, as in the case of thermophilic bacteria.

5. Preparation of high dilutions for the separation of the cells before plating.<sup>20</sup>

6. Isolation of an individual colony. This can be accomplished either (a) by the picking of surface colonies from agar or gelatin plates or slants in large tubes, kept under anaerobic conditions; (b) by picking colonies from deep agar tubes.<sup>21</sup> The last procedure is the easiest and most reliable in the process of separation of pure cultures of anaerobic bacteria from all accompanying forms.

<sup>18</sup> Dorner, 1924 (p. 172).

<sup>19</sup> Ankersmit, P. Centrbl. Bakt. I, Orig., 39: 359-574, 687. 1905; 40: 100-118; Choukevitch, J. Ann. Inst. Past. 25: 247. 1911.

<sup>20</sup> Stoddard, J. L. Jour. Am. Med. Assn. 79: 906. 1918.

<sup>21</sup> Burri, R. Centrbl. Bakt. II, 8: 533-537. 1902.

7. Finally the isolation of single cells either by the India ink method,<sup>22</sup> by the method of Barber, or by one of the microscopic methods.<sup>23</sup>

A detailed study of the various methods used for the isolation from surface colonies is given elsewhere.<sup>24</sup>

In general, plates or large agar slants containing the proper culture media are streaked out and placed either in vacuo, in hydrogen, carbon dioxide, or in an atmosphere from which the oxygen is removed by means of sodium pyrogallate. To produce discreet colonies, the agar plates or slants must be dried before inoculating, but too much drying of the medium is injurious. The slants or plates are streaked out with a loopful of the material taken from the enriched culture or using a dilution of it. The plates are immediately placed in the atmosphere of the neutral gas; the agar may also be placed into the upper part of a Petri dish, which is then covered directly with the sterile inverted lower half of the dish and the whole covered with a larger Petri dish.<sup>25</sup>

The deep colony procedure, used first by Liborius for the isolation of anaerobic bacteria, has been preferred by a number of workers. The selection of a suitable medium for this purpose is essential; the medium should be clear and transparent and enough dilution tubes should be used. Some actively growing anaerobes will grow through the agar as if it was a broth; this "permeating growth" will contaminate the other colonies. The deep tubes of sterile agar are placed in boiling water till the agar is melted, tubes are shaken to remove air, and agar cooled down to 45°. Long boiling is inadvisable, since the cotton becomes saturated with moisture. Three tubes are employed for ordinary purposes of dilution, but for new material or for weakly growing organisms among rapidly growing forms, more tubes may be used. Tube 1 is inoculated with one loopful of the enriched culture or soil suspension. The tube is then shaken, and transfer is made by means of a sterile pipette (a Pasteur pipette may be used), previously flamed, into tube 2. The

<sup>22</sup> Burri, 1909 (p. 55); Krause-Uhlenhut's *Handbuch der mikrobiologischen Technik*. 2: 329. 1923.

<sup>23</sup> Barber, 1911-1920 (p. 55). Kendall, A. I., Cook, M. and Ryan, M. *Jour. Inf. Dis.* 29: 227-234. 1921. Holker, J. *Jour. Path. Bact.* 22: 28. 1919; 23: 192-195. 1920.

<sup>24</sup> von Hibler, E. *Untersuchungen über die pathogenen Anaeroben*. Jena. 1908; Besson, A. *Practical bacteriology, microbiology and serum therapy*. London, 1913; Lentz, O. *Friedberger und Pfeiffer's Lehrbuch der Mikrobiologie*. Jena, 1919, p. 370.

<sup>25</sup> Marino, F. *Ann. Inst. Past.* 21: 1005. 1907; Ogata, M., and Takenouchi, M. *Centrbl. Bakt.* I, 73: 75-77. 1914.

inoculum is placed throughout the length of the agar; care is taken not to blow air into the agar in the tube; the latter is then shaken. The pipette is flamed and, by means of it, some of the agar from tube 2 is transferred to tube 3, which is also shaken. The tubes are plugged with cotton, as ordinary aerobic tubes, and incubated aerobically at 25° to 28°. For actively growing species, 12 to 24 hours' incubation are sufficient; for slow growing forms, such as *Bac. amylobacter*, 4 to 8 days may be required. The colonies are examined, by means of a hand lens, for permeating growth and aerobic organisms. Final isolation is made from the colonies of the mixed culture. The tube and colonies to be transferred are selected. A plain glass or metal rod, sterilized in the flame and cooled, may be used to pierce the agar to the bottom of the tube, so as to admit air and allow the expulsion of the unbroken agar from the tube upon a sterile half of a Petri dish. The agar tube may also be placed for a second or two into warm water so as to separate the agar from the walls of the tube. The agar cylinder is then cut up into fine slices by means of a sterile scalpel; the desired colony is selected, either with the naked eye or using the microscope, and the agar is carefully cut away from it. A transfer is then made, by pricking the colony with a fine sterile platinum needle; to deep tubes with sterile agar or to agar slants or liquid media, which are then incubated in an oxygen-free atmosphere.

When single cells are separated from one another to obtain pure cultures, it is better to isolate the spores rather than vegetative cells, since these give a much larger number of successful cultures. A medium somewhat more acid than the optimum (as pH 6.0) is more favorable for the germination of the spores. Semi fluid media (containing 0.1 to 0.2 per cent agar) are preferable to liquid media, since the presence of a colloid greatly hastens the germination of the bacterial spores.<sup>26</sup>

*Cultivation of anaerobes.* There are a number of methods available for the cultivation of anaerobes, these methods being largely concerned with the reduction of the oxygen tension; some of these have been referred to already previously.

<sup>26</sup> Further information on the isolation of anaerobic bacteria is given by Lantzs, 1921 (p. 544); Kürsteiner, 1907 (p. 170); Veillon, A., and Mazé, P. *Compt. Rend. Soc. Biol.*, **68**: 112. 1910; Northrup, Z. *Jour. Bact.* **1**: 90-91. 1916; Hort, E. C. *Jour. Hyg.* **18**: 361. 1920; Fuhrmann, F. and Pribram, E. *Abderhalden's Handb. biol. Arb. Methoden.* **XII**: 483-702. 1924; Löwi, E. *Centrbl. Bakt. I, Orig.*, **82**: 493-496. 1919.

I. Cultivation in the absence of oxygen:<sup>27</sup>

1. Mechanical protection against the atmospheric oxygen. The use of large volumes of freshly-boiled liquid media placed at a high level; also the process of covering the media with a layer of liquid petrolatum or other inert oil has been known since Pasteur. A layer of solid medium can be placed in a Petri dish, then inoculated with anaerobic bacteria and covered with a solution of agar (1.2 to 1.5 per cent) in distilled water. This layer of agar, in covering the medium, prevents sufficiently the admission of oxygen. The solid medium may also be placed in the upper part of a Petri dish, then covered with the lower part, placed into the upper part. Solid medium may also be placed in deep layers in ordinary containers, then inoculated with a long platinum loop reaching to the bottom of the container. The agar can be taken out from the deep tube, by stabbing to the bottom a sterile glass or metallic tube, 2 mm. in diameter, so as to admit air.<sup>28</sup>

2. Cultivation of anaerobes in vacuo. This method was also proposed by Pasteur and consists in placing the medium in a tube with a capillary end, inoculating, pumping out the air, then sealing the end. Petri dishes can also be placed in an ordinary desiccator, from which the atmosphere is then pumped out. The method described by Meyer<sup>29</sup> can be used for the cultivation of bacteria at different partial oxygen tensions.

3. Absorption of oxygen from the atmosphere. The most common method of absorption of oxygen from the atmosphere is carried out by means of alkaline pyrogallate solution introduced by Buchner.<sup>30</sup> A mixture of equal portions of 10 per cent solutions of pyrogallol and KOH are often used, or 5 per cent solution of the first and 12.5 per cent of the second. The method of Buchner was modified for liquid media.<sup>31</sup> The sterile cotton plug is pushed into the tube; 1 cc. of 20 per cent pyrogallol and 1 cc. of 20 per cent KOH are placed upon it, the tube is then closed with a rubber stopper. An alkaline pyrocatechin  $\text{FeSO}_4$  solution to be used as a sensitive reagent for determining traces of oxygen has been suggested.<sup>32</sup> The following method is very convenient:<sup>33</sup> About 15 to 20 cc. of agar medium is placed in a large tube, about 1 inch in diameter, the tube is plugged with cotton and sterilized, then slanted. Immediately after inoculation, the cotton plug is pressed deeply into the tube, about 1 to 2 inches above the tip of slant. One cubic centimeter of a 20 per cent solution of pyrogallol (or a tabloid containing 0.13 gram of the acid) and 0.25 cc. of a 40 per cent solution of KOH are poured upon the plug, the tube closed with a rubber stopper, turned upside down and placed in the incubator.

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<sup>27</sup> Application of physical and chemical principles in the cultivation of obligate anaerobic bacteria is discussed in detail by Hall, I. C. *Jour. Bact.* 17: 255-301. 1929.

<sup>28</sup> Burri, R., Staub, W. and Hohl, J. *Schweiz. Milchztg.* 45: nos. 78, 83. 1919.

<sup>29</sup> Meyer, 1906 (p. 169).

<sup>30</sup> Buchner, H. *Centrbl. Bakt.* 4: 149. 1888.

<sup>31</sup> Wright, J. H. *Centrbl. Bakt.* I, 29: 61. 1901.

<sup>32</sup> Binder, K. and Weinland, R. F. *Ber. deut. chem. Gesell.* 46: 255-259. 1913.

<sup>33</sup> Buchanan, R. M. *Centrbl. Bakt.* I, Orig., 74: 526-527. 1914.

For Petri dishes, a combination of evacuation and absorption of oxygen may be utilized. Ten per cent solution of KOH is poured upon the bottom of a desiccator and an open Petri dish containing dry pyrogallol is placed in it. The dishes containing fresh medium and inoculated are then placed into the desiccator. The latter is covered and evacuated. The desiccator is then carefully turned so as to mix the alkali with the pyrogallol. Since this takes place in the presence of traces of oxygen it browns only slightly. If the cover is not tight, the admission of oxygen is readily indicated by the rapid browning of the mixture. A beaker with water may be placed in the desiccator to prevent the rapid drying out of the media.<sup>34</sup>

Various other methods for the physical or chemical absorption of the oxygen from the atmosphere have been used; they are based upon the addition of organic or inorganic substances, possessing a strong reducing power, to the medium or outside of the medium in a gas-tight vessel. These include ferrous sulfate, sodium sulfide, ammonium sulf-hydrate, sodium sulfite, ferro-ammonium sulfate, phosphorus; glucose, sodium formate, pyrocatechin, indigo-carmin; metallic iron and zinc; various tissues, pieces of potato, carrot, fresh yeast, etc. These treatments are often accompanied by a partial vacuum. The plates or tubes may be placed in a container to which a quantity of freshly cut potatoes is added, then covered with a bell jar.

4. Replacement of air by an indifferent gas. Hydrogen, carbon dioxide, nitrogen, and other inert gases may be used for this purpose. A tube,<sup>35</sup> flask or desiccator<sup>36</sup> supplied with a two-holed rubber stopper can be used for this purpose. When all the air is replaced by the inert gas, the tubes are sealed.

## II. Cultivation in the presence of oxygen:

5. Cultivation of anaerobes in the presence of aerobic organisms. This method approaches nearest to what takes place in nature than any of the other methods. By cultivating an anaerobic spore forming organism with an aerobic non-spore former, like *Bact. prodigiosum*, it is easy to obtain a pure culture of the former by pasteurization. This method has only a limited application in the study of pure cultures. Beijerinck<sup>37</sup> employed the fungus *Oidium lactis* to eliminate the last traces of oxygen from the atmosphere. A combination of two of the above processes may be used.

The media used for the isolation and cultivation of anaerobic bacteria depend upon the specific organisms. Certain special methods may also be used. Among these, gelatin and milk have played an important part. By inoculating milk with a small quantity of soil, a certain type of butyric acid bacteria can be readily

<sup>34</sup> Rockwell, G. E. Jour. Inf. Dis. 35: 581-586. 1924.

<sup>35</sup> Fränkel, C. Centrbl. Bakt. 3: 735, 763. 1888; Petri, R. J. and Maassen, A. Arb. K. Ges. Amt. 8: 314. 1893.

<sup>36</sup> Botkin, S. Ztschr. Hyg. 9: 383. 1890; Novy, F. G. Centrbl. Bakt. 16: 566. 1894; Richardson, A. C. and Dozler, C. C. Jour. Inf. Dis. 31: 617-621. 1922.

<sup>37</sup> Beijerinck, M. W. Proc. Sec. Sci. K. Akad. Wettensch. Amsterdam, 21: 1219-1226. 1919.

demonstrated. This is not a medium for enrichment of anaerobes, as for differential purposes. Various protein and glucose media can be used. The medical bacteriologists have made extensive use of brain and blood agar media. To demonstrate the presence of certain organisms, specific media may have to be used. To demonstrate the presence of *Bac. amylobacter*, nitrogen-free glucose (2 per cent) agar placed in a deep tube is inoculated with a soil suspension; if quantitative results are wanted, various dilutions are employed (the soil suspension may be previously heated, in a water bath, at 80° for 10 minutes, whereby only the number of spores is obtained). The tubes are closed with rubber stoppers (when the culture is to be isolated, a surface layer of sublimate agar is used) and incubated at 30°. Gas formation will take place on the second day, breaking up the medium. This and the production of butyric acid will indicate the presence of the organism; the colonies are lens-shaped. Microscopic examination of the culture can be made by staining with Lugol's reagent. The method of Burri can be used for determining the number of anaerobic bacteria in the soil, not only by establishing the presence of growth in the final dilution, but by actually counting the colonies in the deep tube.

*Classification of soil anaerobes.* Various systems for the classification of anaerobic bacteria and their relation to aerobes have been proposed at different times.<sup>38</sup> But even at the present time, a proper classification of anaerobes, especially of soil forms is lacking. The idea that anaerobic bacteria vary greatly has served further to increase the existing confusion. This led to various exaggerations, such as the existence of only a few anaerobic forms which change into one another, or the making of new genera on the basis of minor physiological differences.<sup>39</sup>

The following system of classification of soil anaerobes may be suggested here merely as a tentative working basis:

I. Bacteria acting primarily upon carbohydrates:

1. Bacteria utilizing largely simple carbohydrates and starches as sources of energy, often referred to as saccharolytic. Here belong the butyric acid bacteria, often classified as one species, *Bac. amylobacter* A. M. et Bred. These decompose sugars with the formation of butyric acid and gas:

- (a) Nitrogen-fixing bacteria—*Clostridium pastorianum* Winogradsky (*Bac. amylobacter* van Tieghem, *Bac. amylocyme* Perdrix, *Bac. butyricus* Botkin, *Granulobacter saccharobutyricum* Beij., *Bac. orthobutyllicus* Grimbert, *Clostridium americanum* Pringsheim, *Bac. amylobacter* A. M. and Bred.) and allied forms found in great abundance in practically all soils. This organism or group was classified by Bergey as *Cl. butyricum*

<sup>38</sup> Hibler, 1908 (p. 174); Bredemann, 1909 (p. 104); Jungano, M. and Distaso, A. Les anaérobies. Paris. 1910.

<sup>39</sup> Heller, H. H. Bot. Gaz. 73: 70-79. 1922; Jour. Bact. 7: 1-38. 1922.

Prazmowski and by Lehman and Neumann as *Bac. pastorianus* (Winogradsky).<sup>40</sup>

- (b) *Bac. welchii* Migula (*Bac. aerogenes capsulatus* Welch and Nutall, *Bac. perfringens* Veillon and Zuber, *Bac. enteritidis sporogenes* Klein), a short rod 4 to 8 by 1 to 1.5 $\mu$ , single or in pairs; non-motile, forming oval, central or excentric spores; encapsulated. Found repeatedly in the soil and in sewage.<sup>41</sup>

2. Bacteria decomposing pectins.

- (a) *Bac. amylobacter* group, which includes the *Clostridium pastorianum* (same as 1a). The forms causing the retting of flax have been described under various names. Here belong the *Plectridium* of Friebes and Winogradsky, the *Clostridium* of Behrens, the *Plectridium pectinovorum* of Störmer, the *Granulobacter pectinovorum* of Beijerinck and van Delden.<sup>42</sup>

- (b) *Bac. felsineus* Carbone.

3. Bacteria decomposing cellulose:

- (a) Anaerobic bacteria decomposing cellulose at ordinary temperatures. Here belong the hydrogen and methane organisms of Omeliansky, the *Bac. cellulosa dissolvens* Khouvine and others.

- (b) Thermophilic cellulose decomposing bacteria—*Clostridium thermocellum* Viljoen, Fred and Peterson.

II. Bacteria acting primarily upon proteins:

1. Strongly proteolytic forms:

- (a) *Bac. sporogenes* Metchnikoff (No. 58, Pl. IX), a motile, flagellated, gram positive bacillus, with rounded ends, 3 to 7 by 0.6 to 0.8 $\mu$ ; one of the strongest proteolytic bacteria known; it decomposes proteins with the formation of gas, a darkening of the medium and production of a pronounced odor; the sub-terminal spores are formed readily. Found abundantly in soil, manure, street dust and sewage.

- (b) *Bac. oedematis maligni* Koch (*Vibrio septique* Pasteur), found in the intestines of man and in the soil.<sup>43</sup>

- (c) *Bac. putrificus* Bienstock, a motile, flagellated bacillus, forms terminal oval spores and has weak saccharolytic and strong proteolytic properties. Milk is gradually digested, without rapid coagulation.

<sup>40</sup> Further information on the classification of the anaerobic bacteria acting primarily upon carbohydrates is given by Donker, H. J. L. Bijdrage tot de kennis der Boterzuur-, Butylalcohol- en Acetongistingen. Delft. 1926.

<sup>41</sup> Klein and Houston. Rept. Med. Officer, Local Govt. Board, London, 1898-1899, 318; Greer, F. E. Amer. J. Publ. Health, 15: 860-867. 1925.

<sup>42</sup> Ruschmann, G. and Bavendamm, W. Centrbl. Bakt. II, 64: 340-394. 1925.

<sup>43</sup> Gt. Britain National Health Ins. Joint Comm., Medical Research Committee. Special Reports, Series No. 39. 1919.



- (d) *Bac. histolyticus* Weinberg and Seguin, 3.0 to 5.0 by 0.5 to 0.7 $\mu$ , occurring singly or in pairs, motile by peritrichous flagella; spores oval excentric. This organism has been isolated from the soil by Peterson and Hall.<sup>44</sup>
  - (e) *Bac. botulinus* van Ermengem, large rods with rounded ends; oval, subterminal spores. The natural habitat of this organism has been found in virgin and cultivated soils, mountain and forest soils,<sup>45</sup> throughout the world.
2. Weakly proteolytic organisms:
- (a) *Bac. bifermentans* Tissier and Martelly, non-motile bacillus, with large central, oblong to oval spores.
  - (b) *Bac. tetani* Nicolaier, 4 to 8 by 0.4 to 0.6 $\mu$ ; motile by means of peritrichous flagella; unable to utilize carbohydrates, introduced into the soil with the manure.<sup>46</sup> Its occurrence in the soil has been demonstrated<sup>47</sup> in many of the samples examined.

Various other anaerobic bacteria which are weakly proteolytic, but are capable of attacking different carbohydrates, with the formation of gas have been isolated either directly from the soil or from other sources, which may indicate a soil habitat, such as *Bac. chauvoei*. A detailed study of the various anaerobic bacteria, including *Bac. sporogenes*, *Bac. histolyticus* and others, secured from wound infections and probably coming in most cases originally from the soil has been made by Weinberg and Seguin.<sup>48</sup>

### III. Bacteria obtaining their oxygen from inorganic salts:

- 1. Bacteria reducing nitrates.
- 2. Bacteria reducing sulfates.

Anaerobic organisms may occur in the soil in great abundance; Ucke<sup>49</sup> found a garden soil to contain 13½ million cells of anaerobic bacteria and 500,000 spores per 1 gram of soil. In some cases individual species are found in the soil in great abundance. Kürsteiner, for example, found as many as 1 million and more cells of *Bac. putrificus* per 1 gram of soil. Out of 200 samples of Swiss soils examined, only seven

<sup>44</sup> Peterson, E. C. and Hall, I. C. Proc. Soc. Exp. Biol. Med. 20: 502-503. 1923.

<sup>45</sup> Tanner, F. W. and Dack, G. M. Jour. Inf. Dis. 31: 92-100. 1922; Dubovsky, B. J. and Meyer, K. F. Jour. Inf. Dis. 31: 501-540, 541-555, 556-558, 559-594, 595-599, 600-609, 610-613. 1922; Hall, I. C. and Peterson, E. C. Jour. Bact. 9: 201-209. 1924.

<sup>46</sup> Noble, W. Jour. Inf. Dis. 16: 132-141. 1915.

<sup>47</sup> Dubovsky, B. J. and Meyer, K. F. Jour. Inf. Dis. 31: 614-616. 1922.

<sup>48</sup> Weinberg, M. and Seguin, P. La gangrène gazeuse. Masson & Cie. Paris. 1918.

<sup>49</sup> Ucke, A. Centrbl. Bakt. I, 23: 996-1001. 1898.

did not contain *Bac. amylobacter*. The number of colonies formed on artificial media is considerably less than the total number of organisms actually present in the soil; this is brought out by the results of Dorner,<sup>50</sup> who found that out of 1000 spores of *Bac. amylobacter* present in a medium, only 3 germinated and developed into colonies, while out of 1000 vegetative cells, 45.1 produced colonies.

By the use of the dilution and selective culture method, Dügge<sup>51</sup> found 1000 to 1,000,000 anaerobic butyric acid bacteria per gram of soil, 0 to 1000 anaerobic cellulose-decomposing bacteria, 100 to 1,000,000 anaerobic nitrogen-fixing bacteria, from 100 to 1,000,000 anaerobic protein-decomposing bacteria and 100 to 1,000,000 pectin-decomposing bacteria. By the deep tube method, only between 19,000 and 900,000 anaerobic bacteria were found per gram of soil. This is due to the fact that no single solid medium can be devised which would be favorable for the development of all anaerobic bacteria.

Anaerobic bacteria take an active part in the composting of manure in the heap, whenever there is an insufficiency of aeration. The so-called phenomenon of "putrefaction" is chiefly a result of the decomposition of protein substances under anaerobic conditions, due to incomplete oxidation as a result of insufficient aeration. The absence of air in the deeper piles of manure, the slightly alkaline reaction and the presence of large amounts of undecomposed substances make conditions favorable for the development of anaerobic bacteria.<sup>52</sup> Various anaerobic urea bacteria and thermophilic organisms<sup>53</sup> also find conditions in the composting manure heap favorable for their development. Well rotted horse manure contains spore-forming, anaerobic thermophilic bacteria;<sup>54</sup> the limiting temperature for their growth was found to be 60° to 65°C. and the thermal death point 110° to 120°C. Some of these organisms were found to be actively proteolytic. No growth took place at room temperature. A number of anaerobic spore-bearing bacteria are no doubt brought into the soil with the feces in great abundance; certain types have actually been demonstrated in intestinal excreta.<sup>55</sup>

*Physiological activities of anaerobic bacteria.* It is impossible to dis-

<sup>50</sup> Dorner, 1924, (p. 172).

<sup>51</sup> Dügge, 1921 (p. 35).

<sup>52</sup> Severin, S. A. Centrbl. Bakt. II, 1: 799-817. 1895; 3: 628-633, 708. 1897. Zhur. Opit. Agron. 1: 463-489. 1920.

<sup>53</sup> Geilinger, 1917 (p. 165); Veillon, R. Ann. Inst. Past. 36: 422-438. 1922.

<sup>54</sup> Damon, S. R. and Feiber, W. A. Jour. Bact. 10: 37-46. 1925.

<sup>55</sup> Kahn, M. C. Jour. Inf. Dis. 35: 423-478. 1924.

cuss the physiological activities of the various obligate anaerobic bacteria, since they differ greatly in the nature of their metabolism. Those that obtain their energy from cellulose, those that can obtain their nitrogen from the elementary form, those that can utilize nitrate or sulfate oxygen, and those that produce foul odors from complex proteins have a distinct physiology from one another and cannot be considered under one heading, merely because they are similar in their requirements of oxygen tension. They usually have an optimum range of hydrogen-ion concentration at pH 6.0 to 8.2 with a limiting range of pH 5.0 to 9.0; the spores germinate better at a higher acidity, with an optimum at pH 6.0 to 7.2.<sup>56</sup>

While aerobic bacteria produce largely carbon dioxide among the volatile gases, the anaerobic bacteria are characterized by the production of a number of other gases. It is sufficient to mention hydrogen and methane, as a result of decomposition of carbohydrates, hydrogen sulfide as a result of reduction of sulfates, elementary nitrogen and oxides of nitrogen as a result of reduction of nitrates, and various amines, elementary nitrogen and oxides of nitrogen, hydrogen sulfide, mercaptans and thioether as a result of decomposition of proteins. It is necessary to be able to measure these and determine them quantitatively, especially since they are often of great economic importance when a soil is water-logged for a longer or shorter period of time. The bacteria are grown on suitable media (specific for the various organisms) under anaerobic conditions, in tubes or bottles connected with a manometer. The tubes may also be placed in a Novy jar used as a respiratory chamber.<sup>57</sup> The growth may be carried on in an atmosphere of pure gas, such as  $N_2$ ,  $H_2$ ,  $CO_2$ . By using a compensation manometer, the pressure changes taking place within the culture tube or jar can be observed constantly, these changes indicating the periods of active growth followed by the cessation of growth and respiration. The samples of gas are withdrawn directly into a burette or first into a sampler, then into a modified Henderson-Haldane or other suitable apparatus.

The volume of the gas to be analyzed is first measured; the gas is then passed back and forth into 10 per cent KOH solution to absorb the  $CO_2$ , which is determined by difference in the volume of gas. The gas, freed from  $CO_2$ , is passed into

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<sup>56</sup> Dozler, C. C. Jour. Inf. Dis. **35**: 105-133. 1924.

<sup>57</sup> Novy, F. G., Roehm, H. R. and Soule, M. H. Jour. Inf. Dis. **36**: 109-167. 1925.

an alkaline pyrogallate solution (or sticks of yellow phosphorus in water) to absorb the oxygen; the latter is determined also by the difference in volume of the gases. The estimation of hydrogen, methane and other combustible gases is carried on in a combustion chamber over heated platinum, in the presence of oxygen (or air as a source of oxygen). By measuring the amount of  $\text{CO}_2$  formed in combustion, it is possible to calculate the amount of methane and other hydrocarbons present in the gas mixture; the amount of hydrogen is then determined by the difference between the loss due to combustion and the methane present. The amount of oxygen absorbed in the combustion is obtained by calculation or by the difference between the oxygen added and that remaining, as determined by absorption in the pyrogallate solution. The  $\text{CO}_2$  present in the medium (liquid) is aerated into standard  $\text{Ba}(\text{OH})_2$  solution, then titrated.

Oxides of nitrogen are determined by combustion in the platinum spiral before oxygen (or air) is admitted, in the presence of hydrogen. The contraction in volume serves as an index of  $\text{N}_2\text{O}$  ( $\text{N}_2\text{O} + \text{H}_2 \rightarrow \text{H}_2\text{O} + \text{N}_2$ ). The oxides of nitrogen may be absorbed from 100 cc. samples of gas in 200 cc.  $\text{M}/50$   $\text{KOH}$  solution, then oxidized to nitrate by adding 5 cc. of 30 per cent hydrogen peroxide. The solution is evaporated to dryness on a water bath and nitrates determined by the phenoldisulphonic acid method.<sup>58</sup> Volatile amines and mercaptans do not occur in great abundance among the decomposition products in the soil, but are found largely in the anaerobic decomposition of manure:<sup>59</sup>  $\text{H}_2\text{S}$  gas can be determined by absorption with acetates of lead and cadmium, or ammoniacal cadmium chloride solution, then titrating the  $\text{CaS}$  with iodine in acid solutions.<sup>60</sup>

Anaerobic bacteria form various acids (acetic, butyric, propionic, formic, lactic), alcohols (ethyl, butyl), and in some cases acetone. Often closely related organisms vary greatly in their metabolic products.

*Soil processes in which anaerobic bacteria take an active part.* Attention has already been called to a number of important physiological processes in the soil, in which anaerobic bacteria take an active part. It is sufficient to indicate that such processes as decomposition of celluloses, pectins and proteins, and the fixation of nitrogen non-symbiotically are as active anaerobically as aerobically. Ammonia formation from proteins is very active under anaerobic conditions.<sup>61</sup> Two maxima were found for nitrogen-fixation in the soil, one under aerobic and another under anaerobic conditions;<sup>62</sup> a higher fixation may actually

<sup>58</sup> Allison, V. C., Parker, W. L. and Jones, J. W. Tech. Paper No. 249, U. S. Bureau of Mines. 1921.

<sup>59</sup> Guggenheim, M. Die biogenen Amine. 1920; Hirsch, P. Borntraeger. Berlin, 1918.

<sup>60</sup> Anderson, B. G. Jour. Inf. Dis. 35: 213-243. 1924.

<sup>61</sup> Löhnis, 1905 (p. 13); Traaen, A. E. Centrbl. Bakt. II, 45: 115. 1916; Murray, T. J. Jour. Bact. 1: 597-614. 1916.

<sup>62</sup> Greaves, J. E. Soil Sci. 6: 163-218. 1918; Lipman and Sharp, 1915 (p. 512).

be obtained anaerobically.<sup>63</sup> The decomposition of cellulose under anaerobic conditions is carried on entirely by bacteria. The phenomena of reduction under anaerobic conditions, especially that of nitrates, may become an important economic factor.

It is important to point out, in this connection, the active rôle which anaerobic bacteria play in the rotting of manure. As a matter of fact, the lowest loss of nitrogen and the most efficient conservation of the important elements of the manure is accomplished by keeping it compact and moist, so as to prevent the action of aerobic fungi and bacteria and stimulate the action of anaerobic bacteria. As far back as 1889, Schloesing<sup>64</sup> pointed out that under anaerobic conditions there is no loss of nitrogen. The gases were found to consist of equal volumes of methane and carbon dioxide, when the manure is incubated at 52°C. Water takes part in the reaction supplying some oxygen for the formation of CO<sub>2</sub> and some hydrogen for the methane. The amount of gas produced per hour rapidly increases until it reaches a maximum on the 6th day, when it begins to diminish. At 42°C., 850 cc. of gas collected from the decomposition of 100 gm. of manure consisted of 713.6 cc. CO<sub>2</sub>, 97.6 cc. methane and 38.8 cc. hydrogen.

Further information on the decomposition of proteins and carbohydrates under anaerobic conditions and on the nature of soil gases is given elsewhere (p. 561). A detailed discussion of the rôle of anaerobic bacteria in the decomposition of organic matter in peat bogs, in the formation of which they play a most important part, is also given elsewhere (p. 657). The nature and activities of various anaerobic bacteria concerned in specific physiological processes are described in the particular sections of this book.

<sup>63</sup> Panganiban, E. H. Phillip. *Agricult.* 12: 63. 1923; *Jour. Amer. Soc. Agron.* 17: 1-31. 1925.

<sup>64</sup> Schloesing, 1889 (p. 62).

## CHAPTER VIII

### BACTERIA DECOMPOSING CELLULOSE AND OTHER COMPLEX CARBOHYDRATES AND HYDROCARBONS

*Microorganisms decomposing cellulose in nature.* Among the microorganisms concerned in the decomposition of different constituents of plant and animal tissues those capable of breaking down true cellulose have attracted considerable attention, due to the fact that this compound makes up a large part of the bulk of the organic matter added to the soil, and also because of the fact that the organisms concerned in this process are more or less specific. Many bacteria are capable of existing with cellulose as the only source of energy material, while some cannot even utilize any other source of energy. Organisms capable of decomposing cellulose are found among various groups of fungi, among the actinomyces and among aerobic and anaerobic bacteria.

The general impression prevailed until recent years that the anaerobic bacteria are the most important agents in the decomposition of cellulose in nature. More recently, however, considerable evidence has been submitted<sup>1</sup> to prove that the aerobic bacteria are of greater importance than the anaerobic bacteria in the decomposition of cellulose in soil and in composts; under other conditions, as in peat bogs, the anaerobic bacteria are very active. In addition to the true *anaerobic* and *aerobic* bacteria, certain special groups of cellulose-decomposing organisms are frequently recognized, namely the *thermophilic* bacteria, probably active in the decomposition of cellulose in manure and in soil under certain special conditions, and the so-called *denitrifying* bacteria, active only in the presence of nitrates and also under certain specific conditions.

*Anaerobic cellulose-decomposing bacteria.* Mitscherlich<sup>2</sup> observed in 1850 that, in the rotting of potatoes in water, the cell walls are destroyed while the starch accumulates at the bottom of the container. He ascribed this action to the vibrios which were present in abundance in

<sup>1</sup> Pringsheim, H. Die Polysaccharide. 3 Aufl. Springer. Berlin. 1931; Rippel, A. Ztschr. angew. Bot. 1: 78-97. 1919; Waksman, S. A. and Skinner, C. E. Jour. Bact. 12: 57-84. 1926; Winogradsky, S. Ann. Inst. Past. 43: 549-633. 1929.

<sup>2</sup> Mitscherlich. Monatschr. K. Akad. Wiss. Berlin. 1850, 102-110.

the water. Van Tieghem<sup>3</sup> described in detail a species of *Amylobacter* previously found to occur in decomposing plant tissues and staining blue with iodine; it decomposed young plant tissues with the formation of butyric acid, carbon dioxide and hydrogen. This organism was not a species in the true sense of the word, but a collective form; it is doubtful whether it decomposed pure cellulose at all, so that it could hardly deserve the term "cellulose organism."<sup>4</sup> Since cellulose forms an important constituent of manure, attention has been directed chiefly towards cellulose decomposition in the rotting of manure.

Omeliansky<sup>5</sup> was the first to establish definitely the connection between the activities of microorganisms and the decomposition of cellulose.

The following medium was employed:

K <sub>2</sub> HPO <sub>4</sub> .....	1.0 gram	MgSO <sub>4</sub> .....	0.5 gram
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> or		NaCl.....	trace
(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> }	1.0 gram	Distilled water.....	1000 cc.

The ammonium salt may be replaced by 0.5 per cent asparagine or 0.1 per cent peptone. Some chalk and pure filter paper were placed in long-necked bottles, which were then filled with the medium to the stopper. The flasks were inoculated with horse manure or river mud and incubated at 34° to 35°. After a considerable period of incubation (usually more than a week), gas production set in. The paper became covered with specks; these were the places where the decomposition of the cellulose began. At the end of the growth period (active fermentation), which was accompanied by abundant gas formation, there remained only a part of the paper, half rotted and entirely changed in appearance. This residue fell apart at the slightest touch. The white color of the paper had changed into yellow-brown, the medium also was colored, and the odor of the medium was that of rotten cheese. When precipitated cellulose was used in place of filter paper, the reaction was more rapid.

Omeliansky found that the gases liberated in the decomposition of the cellulose contained either hydrogen or methane. On further study, he observed that these two gases seemed to be produced by two different organisms: when the inoculum was added without preliminary heating, methane formation took place; when the inoculum was heated for 15 minutes at 75°C. conditions favored the development of bacteria which

<sup>3</sup> Van Tieghem, P. E. L. Compt. Rend. Acad. Sci. 68: 205-210; 89: 5-8, 1102-1104. 1879; Bull. Soc. Bot. France, 24: 128-135. 1877; 26: 25. 1879; 28: 243-245. 1887.

<sup>4</sup> Omeliansky, W. L. Lafar's Handb. tech. Mykol. 3: 245-268. 1904.

<sup>5</sup> Omeliansky, W. L. Centrbl. Bakt. II, 8: 193-201, 225-231, 257-263, 289-294, 21-326, 353-361, 385-391, 1902; 11: 369-377, 1904; 36: 472-473. 1913.

produced hydrogen in the decomposition of cellulose. The spore of the methane-forming organism was found to germinate earlier than that of the hydrogen form. When the culture was transferred, the former organism predominated and the latter could finally be entirely eliminated. By heating the inoculum of a young culture, the vegetative cells produced from the spores of the methane form, which had already germinated, were killed, while the ungerminated spores of the hydrogen form survived and proceeded to develop. If this process of heating the culture at an early stage of development was repeated several times, the hydrogen form could be obtained free from the methane form.

The hydrogen organism (*Bac. fossicularum* L. et N.) was found to form thin, straight rods (4 to 8 by  $0.5\mu$ ) in young cultures. With age of culture the cells became longer until they reached a length of 10 to  $15\mu$ , without increasing in thickness and without forming chains. The cells were often slightly curved, sometimes even spiral-like, especially on precipitated cellulose. At a later stage, one end of the cell swelled up gradually and took the appearance of an oblong and then of a round body. A perfectly round spore developed in this swelling, filling all the space, with a diameter not exceeding  $1.5\mu$  when ripe. After some time, the spore was liberated by the breaking up of the mother cell. Old cultures, in which the decomposition of the paper is well advanced, showed only spores with a slight admixture of vegetative forms which were usually in the stage of forming spores. The spore-containing cultures could be stained with a double stain of carbol fuchsin and methylene blue. The organism was never colored blue with iodine and, therefore, lacked the important characteristics of *Bac. amylobacter*. By repeated transfers on enrichment culture media, a microscopically pure culture of the organism could be obtained, especially if the inoculum was heated for 20 minutes at  $90^{\circ}\text{C}$ . to kill all non-spore forming contaminations. But all repeated attempts to cultivate the organism on solid media failed. This prevented a detailed study of its metabolism.

The methane organism (*Bac. methanigenes* L. et N.) was quite similar to the hydrogen organism, but somewhat thinner and more gently contoured. By several transfers and on heating the inoculum, a culture was obtained which seemed microscopically pure. Chains were never formed in a young stage and the cells had a tendency to curve slightly. The spores were smaller than those of the hydrogen form, being  $1\mu$  in diameter. Iodine did not give a blue color. Morphologically both organisms could be classified as one species, while, physiologically, they were distinctly different. Attempts to cultivate this organism on solid media and obtain a pure culture also failed.



Kellerman and associates<sup>6</sup> could not confirm the results of Omeliansky. They isolated from Omeliansky's cultures an aerobic cellulose-decomposing organism and suggested, therefore, that the cellulose was decomposed by aerobic bacteria, while the accompanying anaerobic forms produced gas from the products of decomposition of the cellulose by the former. Khouvine<sup>7</sup> claims to have succeeded in isolating from the intestine of man an obligate anaerobic organism, *Bac. cellulosaе dissolvens*, capable of decomposing cellulose very vigorously, especially in mixed culture. The vegetative cells were 2.5 to 12.5 $\mu$  long, and did not form any flagella; the spores were 2.5 by 2 $\mu$  in size. It was cultivated upon a medium containing fecal matter as a source of nitrogen. The spores were killed only on boiling for 45 to 50 minutes. Cellulose was

<sup>6</sup> Kellerman et al., 1912-1914 (p. 192).

<sup>7</sup> Khouvine, Mme. Y. Digestion de la cellulose par la flore intestinale de l'homme. Cour D'Appel. Paris. 1923.

## PLATE VIII

### CELLULOSE AND PECTIN-DECOMPOSING BACTERIA

64. (x), Hydrogen-forming anaerobic cellulose-decomposing bacteria, and (y), methane-forming anaerobic cellulose-decomposing bacteria: a, young cells; b, spore formation; c, ripe spores,  $\times 660$  (from Omeliansky).

65. *Bac. cellulosaе dissolvens*, showing bacteria attached to the cellulose fiber, by their non-sporulating extremities (from Khouvine).

66. Holes in paper produced by *Cytophaga Hutchinsoni*, grown in Petri dish culture upon  $\text{NaNO}_3$ -mineral salt agar with filter paper superimposed, natural size (from Hutchinson and Clayton).

67. *Cytophaga Hutchinsoni*, young culture on filter paper placed in tube; typical incurvation of thread forms (from Hutchinson and Clayton).

68. *Cytophaga Hutchinsoni*, formation of pre-sporoid stage with double granules (from Hutchinson and Clayton).

69. *Bact. fimi*, 15-day old colonies on cellulose agar plate, at 30°C. (from McBeth and Scales).

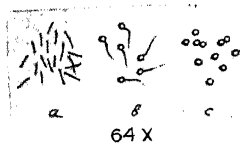
70. *Bact. fimi*, vegetative cells from 24-hour culture on nutrient agar, stained with carbol-fuchsin,  $\times 660$  (from McBeth and Scales).

71. *Bac. cytaseus*, 15-day old colonies on cellulose agar plate, at 30°C. (from McBeth and Scales).

72. *Bac. cytaseus*, nine day old culture at 30°C., showing spore formation; aqueous fuchsin stain,  $\times 660$  (from McBeth and Scales).

73. *Clostridium thermocellum*, a thermophilic cellulose decomposing bacillus. A 48 starch-agar culture at 65°C. stained with carbol fuchsin for 5 minutes, at 100°C., showing free spores, sporangia and vegetative rods (from Viljoen, Fred and Peterson).

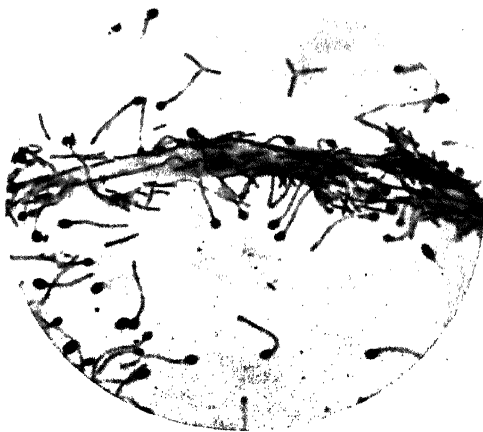
74. *Granulobacter pectinovorum* (after Beijerinck).



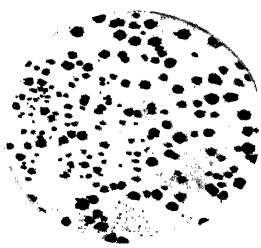
64X



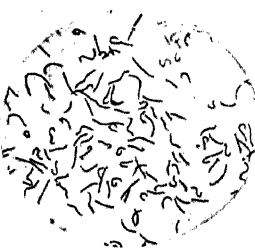
64Y



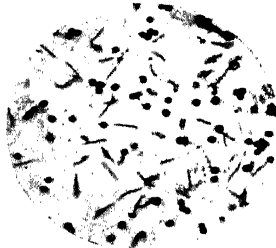
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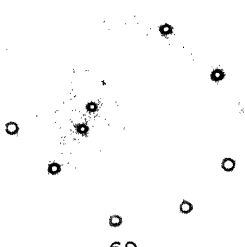
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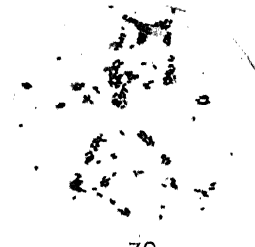
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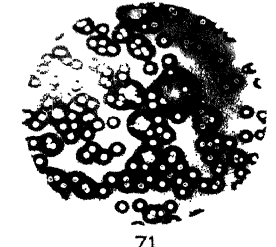
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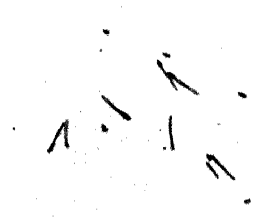
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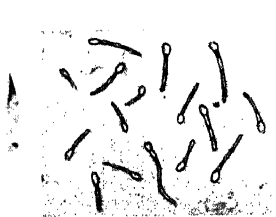
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decomposed at 38° to 51°C., without any distinct optimum. When the oxygen tension of the atmosphere was above 12 mm. mercury, no growth took place. The cells of the specific bacterium clung to the paper, so that the contaminating forms could be removed by washing the paper with sterile salt solution. Sixty per cent of the cellulose decomposed was accounted for by the carbon dioxide, hydrogen, ethyl alcohol, acetic and butyric acids, and a brown pigment. The presence of other bacteria greatly stimulated the power of this organism to decompose cellulose. This anaerobic bacterium was found to be also

TABLE 17

*Summary of characteristics of three anaerobic cellulose decomposing bacteria*

	BACILLUS CELLULOSAE DISSOLVENS— KROUVINE	BACILLUS CELLULOSAM FERMENTANS—WERNER	CLOSTRIDIUM THERMO- CELLUM—VILJOEN, FRED AND PETERSON
Size of cell.....	2.0 x 2 to 12 $\mu$	0.5-0.7 x 1.5-2.7 $\mu$	0.4 x 5.0 $\mu$
Size of spore.....	2.0 x 2.5 $\mu$	1.0-1.2 x 1.5-2.0 $\mu$	0.9 x 0.6 $\mu$
Flagella.....	Absent	Peritrichous	Peritrichous
Nutrient broth with or without glucose.	No growth	No growth	Ring, pellicle and sed- iment; acid and gas with glucose
Nutrient agar.....	No growth	No growth	Small surface and subsurface colonies on starch agar
Potato slant.....	No growth	No growth	Yellow growth, potato browned
Milk.....	No growth	No growth	Acid curd in three days
Carbohydrates de- composed.....	Only cellulose	Only cellulose	Hemicellulose, starch, various hexoses and pentoses

abundantly distributed in the soil, occurring in all soil types, under various conditions.

Werner<sup>8</sup> isolated from the intestinal tract of the larvae of *Potosia cuprea* an anaerobic organism, *Bac. cellulosam fermentans*, capable of attacking rapidly cellulose but no other carbohydrates, thus assisting the insect in its digestive processes. The results of other investigators<sup>9</sup>

<sup>8</sup> Werner, E. Centrbl. Bakt. II, 67: 297-330. 1926.

<sup>9</sup> Hüsslin, A. and Lesser. Ztschr. Biol. 54: 47. 1910; Beckwith, T. D. and Rose, E. J. Proc. Soc. Exp. Biol. Med. 27: 4-6. 1929; Cowles, P. B. and Rettger, L. F. Jour. Bact. 21: 167-182. 1931.

also point to the anaerobic nature of cellulose decomposing bacteria in the digestive tract of horses, cattle, termites, and insect larvae. In general, under anaerobic conditions, cellulose decomposition is carried out entirely by bacteria; the nature of the processes involved is different from that of cellulose decomposition under aerobic conditions. The thermophilic cellulose decomposing bacteria are also anaerobic organisms, decomposing cellulose very actively; due to their specific temperature requirements, they are described separately. In animal digestion of cellulose, *Amylobacter navicula*<sup>9a</sup> was found to be very active.

*Aerobic bacteria.* The first attempt to study cellulose decomposition under aerobic conditions was made by Van Iterson,<sup>10</sup> who described a non-spore-forming bacterium, *Bacillus ferrugineus*, which decomposed cellulose aerobically, in symbiosis with a yellow micrococcus, the latter not decomposing any cellulose when alone.

The following medium was used:

Filter paper.....	2 grams	K <sub>2</sub> HPO <sub>4</sub> .....	0.05 gram
NH <sub>4</sub> Cl (or KNO <sub>3</sub> ,		CaCO <sub>3</sub> .....	2.0 grams
KNO <sub>3</sub> , MgNH <sub>4</sub> PO <sub>4</sub> ,		Tap water.....	100 cc.
peptone).....	0.1 gram		

The medium was placed in Erlenmeyer flasks to a depth of 0.5 to 1 cm., inoculated and incubated at 28° to 35°. Cellulose decomposition was also demonstrated by placing two pieces of filter paper and some powdered ammonium magnesium phosphate in a dish, moistening with a 0.05 per cent solution of K<sub>2</sub>HPO<sub>4</sub> and inoculating with some soil. Yellowish brown spots were produced on the paper in 4 to 5 days; the paper soon became pulpy, and the individual fibers became enveloped in a "micrococcus mucilage." Pure cultures of the organism could never decompose the paper.

Merker<sup>11</sup> described two bacteria, *Micrococcus cytophagus* and *M. melanocyclus*, neither of which was isolated in pure culture, but which were found to be accompanied by a rod-like organism; they decomposed paper partly immersed in the medium with the formation of transparent yellowish spots. A similar organism was studied by Bojanowsky<sup>12</sup> on a silica gel medium, but he also failed to separate the coccus-like form from the rod-shaped form.

In 1918, Hutchinson and Clayton<sup>13</sup> reported the isolation from the

<sup>9a</sup> Causen, P. Centrbl. Bact. II, 84: 20-60. 1931.

<sup>10</sup> Van Iterson, C. Centrbl. Bakt. II, 11: 689-698. 1904.

<sup>11</sup> Merker, E. Centrbl. Bakt. II, 31: 578. 1912.

<sup>12</sup> Bojanowsky, R. Centrbl. Bakt. II, 64: 222-233. 1925.

<sup>13</sup> Hutchinson, H. B. and Clayton, J. Jour. Agr. Sci. 9: 143-173. 1918.

soil of an organism which develops first as a sinuous filamentous cell (3 to 10 by 0.3 to 0.4 $\mu$ ), and which goes through a number of phases, finally terminating in the production of a spherical body or sporoid. This was found to differ in a number of respects from the true spores of bacteria; germination of the sporoid again gave rise to the filamentous form which possesses perfect flexibility and is feebly motile although no flagella were observed. This organism was named *Spirochaeta cytophaga*. More recent investigations (p. 194) established the fact that the spherical body is not a stage in the life cycle of the organism, but merely a contaminant.

This organism is aerobic, with an optimum at 30°C., and is destroyed when kept at 60°C. for ten minutes. It does not grow in nutrient agar or gelatin, and is injuriously affected by concentrations of peptone above 0.025 per cent. As sources of nitrogen, ammonium salts, nitrates, amides and amino acids can be used, with cellulose as the only source of carbon. The soluble carbohydrates are more or less toxic. The following medium was used for its isolation and cultivation:

K <sub>2</sub> HPO <sub>4</sub> .....	1.0 gram	Fe <sub>2</sub> Cl <sub>6</sub> .....	0.01 gram
CaCl <sub>2</sub> .....	0.1 gram	NaNO <sub>3</sub> .....	2.5 grams
MgSO <sub>4</sub> .....	0.3 gram	Cellulose.....	10.0 grams
NaCl.....	0.1 gram	H <sub>2</sub> O.....	1000 cc.

The same organism was found to occur in the soil and capable of decomposing cellulose readily.<sup>14</sup> By the use of the silica gel method, it can be shown to be very abundant in soils receiving applications of farmyard manure and straw.<sup>15</sup>

The gel is prepared by placing a mixture of equal parts of a normal solution of HCl and its equivalent of potassium silicate solution into Petri dishes. After the gel has formed, the dishes are placed in running tap water for 24 hours, then several times in boiled distilled water until free from chlorides. Five grams of ground filter paper are then suspended in 100 cc. of medium containing 5 grams (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 1 gram MgSO<sub>4</sub>, 1 gram KCl, 0.02 gram FeSO<sub>4</sub>, in 1000 cc. distilled water; about 2 cc. portions of the suspension are spread over the surface of each plate and a small amount of CaCO<sub>3</sub> is dusted on. The plates are then placed in the thermostat at 60°C., until the surface of the gel becomes free from excess of liquid. Small particles of soil can then be inoculated directly upon the plate which is then covered and incubated. After 2 to 4 days, yellow and orange growth will be found to develop from the soil into the medium. Transfers are

<sup>14</sup> Löhnis, F. and Lochhead, G. Centrbl. Bakt. II, 58: 430-434. 1923.

<sup>15</sup> Waksman, S. A. and Carey, C. Jour. Bact. 12: 87-95. 1926; Winogradsky, S. 1929 (p. 185).

then made into flasks containing 1 gm. of filter paper and 25 cc. of above solution. The organisms will begin to develop in the form of yellow specks, forming a yellow slimy mass all over the paper in 2 to 4 days. By repeated dilutions, the bacterium can be isolated pure.

The quantitative distribution of *Sp. cytophaga* in soil can also be demonstrated as follows: the soil is diluted with sterile water; 1 cc. portions of the various dilutions are added to test tubes containing a strip of paper partly immersed in 5 cc. of a medium consisting of 0.5 gram  $\text{NaNO}_3$ , 1.0 gram  $\text{K}_2\text{HPO}_4$ , 0.5 gram  $\text{MgSO}_4$ , 0.5 gram  $\text{KCl}$  and 0.01 gram  $\text{FeSO}_4$  in 1000 cc. distilled water. The low nitrogen concentration and the slightly alkaline reaction (pH 7.5) favor the growth of the bacteria and retard the growth of the fungi. At  $28^\circ\text{C}$ , bacterial development will take place within 36 to 72 hours. By the use of this method, as many as 50,000 to 50,000,000 cells of *Sp. cytophaga* were found in 1 gram of soil.<sup>16</sup> The limiting acid reaction for most of the aerobic cellulose-decomposing bacteria is pH 6.0.

In addition to the above organism, various other bacteria capable of decomposing cellulose under aerobic conditions were found in great abundance in soil. Kellerman and associates<sup>17</sup> developed a special culture medium, for which the cellulose was prepared by dissolving paper in Schweizer's reagent, precipitating with hydrochloric acid and washing with water until free from copper and chlorine. The suspension of the precipitated cellulose is then concentrated, so as to contain 0.5 per cent cellulose, and is added to a mineral solution with 1 per cent of agar.

When soil suspensions are directly inoculated upon poured plates of cellulose agar, filamentous fungi develop and tend to overgrow the plate, making difficult the isolation of the bacteria. The sample of soil or manure is first added to sterile flasks containing the cellulose broth or peptone cellulose broth and, as soon as the cellulose shows signs of disintegration, transfers are made upon fresh sterile flasks. This avoids the development of fungi. After several preliminary cultivations upon the liquid enrichment media, the cultures are plated out on the cellulose agar.

<sup>16</sup> Dubos, R. J. Jour. Bact. 15: 223-234. 1928.

<sup>17</sup> Kellerman, K. F. and McBeth, I. G. Centrbl. Bakt. II, 34: 485-494. 1912; Kellerman, K. F., McBeth, I. G., Scales, F. M. and Smith, N. R. Ibid. 39: 502-522. 1914; McBeth, I. G. and Scales, F. M. U. S. Dept. Agr., Bur. Pl. Ind., Bul. 266, 1913; McBeth, I. G. Soil Sci. 1: 437-487. 1916; Scales, F. M. Centrbl. Bakt. II, 44: 661-663. 1915.

The colonies of cellulose-decomposing organisms developing on the plates show a translucent area due to the decomposition of the cellulose in the agar, as well as neutralization of the  $\text{CaCO}_3$  by the acids formed from the ammonium sulfate and cellulose decomposition. Starch agar and finally nutrient agar may be used for the final cultivation of the organisms. The cellulose-destroying bacteria studied by Kellerman and associates were found to grow more rapidly under aerobic conditions, although some anaerobic development has also been observed. They show a more vigorous growth on media containing organic nitrogen (peptone) than inorganic nitrogen. They usually reduce nitrates to nitrites, attack various carbohydrates, and do not form any gaseous products from cellulose or the lower carbohydrates. The paper is disintegrated into fine fibres and a small amount of organic acids is formed.

The following liquid medium was used for demonstrating the dissolution of the filter paper:

Soil extract.....	500 cc.	$(\text{NH}_4)_2\text{SO}_4$ .....	1.0 gram
Distilled water.....	500 cc.	Peptone.....	2.0 grams
$\text{K}_2\text{HPO}_4$ .....	0.5 gram	$\text{CaCO}_3$ .....	excess

All the forms studied were rod-shaped organisms varying in length from 0.8 to  $3.5\mu$ . Only in three species were involution forms observed. Five species produced spores. Twenty-seven of the thirty-six species isolated were motile.

These bacteria are also widely distributed in the soil. When kept under laboratory conditions, for any length of time, especially on nutrient agar media, they undergo marked physiological changes which may include loss of cellulose-decomposing power. Transfers made from the clear zone around the colony of the cellulose-decomposing bacterium growing on the plate decomposed cellulose readily; when the cultures are transferred upon nutrient agar, the organisms lose their cellulose-decomposing power. Löhnis and Lochhead<sup>18</sup> suggested that the thread-like bacterium, described above, plays a prominent rôle in the aerobic decomposition of cellulose, and is accompanied by numerous other cellulose-decomposing bacteria of lower efficiency. This led Omeliansky and Pringsheim<sup>19</sup> to suggest that the organisms isolated by Kellerman, McBeth and Scales are not in themselves cellulose decomposing forms but were present as contaminations; when isolations are attempted on the agar plate, these contaminations are isolated, while the true cellulose decomposing forms are lost.

Gray and Chalmers<sup>20</sup> isolated from the soil an aerobic organism ca-

<sup>18</sup> Löhnis and Lochhead, 1923 (p. 191).

<sup>19</sup> Omeliansky, 1913 (p. 186; Pringsheim, H., and Lichtenstein, S. *Centrbl. Bakt. II*, 60: 309-311. 1923.

<sup>20</sup> Gray, P. H. H. and Chalmers, C. H. *Ann. Appl. Biol.* 11: 324-338. 1924; van der Lek, J. B. *Tijdscha Hyg. Microb.* 3: 276-280. 1929.



pable of decomposing cellulose and liquefying agar (*Microspira agar-liquefaciens*); this bacterium was  $2 \times 0.5\text{--}0.7\mu$  in size, with coccoid forms  $0.5\text{--}0.7\mu$ , motile with a single flagellum. The addition of dextrin, lignin, xylose and certain other sugars stimulated cell development.

A large number of aerobic cellulose-decomposing bacteria were isolated from the soil by Kalnins;<sup>21</sup> twelve of the strains were found to belong to the genus *Vibrio*, four to the genus *Bacterium*, and one to the genus *Bacillus*. The cellulose was decomposed, with inorganic salts or organic compounds as sources of nitrogen. These organisms were able to derive their energy also from different sugars. Reducing sugars were formed in the decomposition of the cellulose. In addition to these, a large number of other aerobic bacteria, capable of decomposing cellulose, have been isolated from the soil, but the identity of many of these forms is very doubtful.<sup>22</sup>

A detailed systematic study of various aerobic cellulose-decomposing bacteria found in the soil has been made by Winogradsky<sup>23</sup> who divided these organisms into three genera:

*Cytophaga*, comprising a group of long, slender, flexible filaments, 3 to  $8\mu$  long and pointed at each end. Motility probable although unknown. Very specific with only cellulose used as a source of energy. The cellulose is changed thereby into a colloidal gel. The paper is colored yellow, orange, rose, red, etc. Four species were described, including *Cytophaga Hutchinsoni*, the organism previously described by Hutchinson and Clayton as *Spirochaeta cytophaga*.

*Cellvibrio*, slender, bent rods with rounded ends, 2 to  $5\mu$  long; actively motile, with one flagellum. Cellulose decomposition is not invariably specific. Cream to ochre colored pigment, readily diffusing. Very abundant, although only two species were described.

*Cellfalcicula*, comprising spindle- or sickle-shaped cells, not exceeding  $2\mu$  in length, with pointed ends. Motile, with one flagellum. Paper stained green and cream-colored, never distinctly yellow, red or orange, as the first two genera are. Specific in respect to cellulose. Three species described.

<sup>21</sup> Kalnins, A. Acta Univ. Latviensis, Lauk. Fak. Ser. I, No. 11. 1930. A detailed study of the spore-forming aerobic cellulose-decomposing bacteria has been made by P. E. Simola. Ann. Acad. Sci. Fenn. A. 34: No. 1. 1931.

<sup>22</sup> Sack, J. Centrbl. Bakt. II, 62: 77-80. 1924; Epstein, A. Bull. Soc. Bot. Genève (2), 11: 191-198. 1920; Distaso, A. Compt. Rend. Soc. Biol. 70: 995-996. 1911; Hopfe, A. Centrbl. Bakt. I, 83: 374-386. 1919; Gescher, N. Faserforschung, 2: 28-40. 1922.

<sup>23</sup> Winogradsky, S. 1929 (p. 185).

Bokor<sup>24</sup> believed to have obtained sufficient evidence that the *Spirochaeta cytophaga* H & C produced thin, branching threads, and is thus related to the Actinomyces. He suggested placing the organism in a genus *Mycococcus* among the Actinomycetaceae, under the specific name of *Mycococcus cytophagus*. Bergey placed, in the third edition of his manual, this organism in the genus *Mycobacterium*, using Wino-gradsky's terminology; in this he was no doubt fully justified. However, the inclusion of the other two genera, namely *Cellvibrio* and *Cellfalcicula* in the same family is totally unjustified, especially, since the rod-shaped cellulose-decomposing bacteria were already placed in this manual in a separate tribe, under the genus *Cellulomonas*.

*Thermophilic cellulose-decomposing bacteria.* The presence in the soil of cellulose-decomposing organisms capable of growing at 60 to 65°C was first demonstrated by MacFayden and Blaxall.<sup>25</sup> The process of cellulose decomposition was accelerated under anaerobic conditions; it was believed to be carried out by the combined action of several organisms. Kroulik,<sup>26</sup> using a medium similar to that employed by Omeliansky, demonstrated the common occurrence of bacteria able to decompose cellulose at 60° to 65°C., particularly in places where cellulose is present in abundance. Both aerobic and anaerobic bacteria were concerned in the process. Two aerobic forms were found in great abundance during the early stages of decomposition, but when isolated on nutrient agar media, they did not decompose cellulose further. Two anaerobic bacteria were isolated which did not grow upon agar.

Various other thermophilic cellulose-decomposing bacteria have also been isolated from the soil.<sup>27</sup> For this purpose a medium consisting of 2 grams  $\text{NaNH}_4\text{HPO}_4 \cdot \text{H}_2\text{O}$ , 1 gram  $\text{KH}_2\text{PO}_4$ , 0.3 gram  $\text{CaCl}_2$ , 5 grams peptone, 15 grams cellulose, 1000 cc. of tap water and excess of  $\text{CaCO}_3$  is inoculated with infusions of rapidly decomposing manure and the cultures are incubated at 65°C. Gas bubbles begin to appear after 18 to 24 hours and the formation of  $\text{H}_2\text{S}$  becomes evident. After 30 to 36 hours, the cellulose pulp is raised to the surface, loses its fibrous structure, and turns brownish-yellow in color. On further transfer, the

<sup>24</sup> Bokor, R. Archiv. Mikrob. 1: 1-34. 1930.

<sup>25</sup> MacFayden, A. and Blaxall, F. R. Trans. Jenner Inst. Prev. Med. 2: 162-187. 1899.

<sup>26</sup> Kroulik, 1913 (p. 151); see also Noack, 1912 (p. 287).

<sup>27</sup> Pringsheim, H. Centrbl. Bakt. II, 38: 513-516. 1913; Langwell, H. and Lymn, A. Jour. Soc. Chem. Ind. 42T: 280-287. 1923; Viljoen, J. A., Fred, E. B. and Peterson, W. H. Jour. Agr. Sci. 16: 1-17. 1926; Tetrault, P. A. Centrbl. Bakt. II, 81: 28-45. 1930.

culture does not form any more  $H_2S$ . The cellulose begins to decompose after 18 hours and the process is completed after 6 to 8 days. When inoculations from individual colonies are made upon glucose agar, the isolated cultures do not decompose cellulose. The culture can be purified by means of deep agar-cellulose tubes. The organism does not grow at 28 to 37°C., makes some growth at 43° to 50°C., but grows best at 65°C. The spores are destroyed at 115°C. in 37 minutes. When grown on common agar media, the power of cellulose decomposition is lost. This is probably due to the loss of some highly oxidizable component, during the process of plating, which is needed to initiate the process of decomposition, or to a change in the physiology of the organism, or to the fact that two or more organisms are necessary to bring about the decomposition of cellulose under those conditions.

A comparative summary of the thermophilic organism (*Clostridium thermocellum*) of Viljoen, Fred and Peterson and the anaerobic cellulose-decomposing organisms of Khouvine and Werner is given in table 17. *Bac. cellulosa* *dissolvens* and *Bac. cellulosa* *fermentans* are unable to grow on any media except those containing cellulose. *Cl. thermocellum* can grow with other sources of energy. The fact, however, that this organism loses its ability to decompose cellulose when grown on other sources of energy would make us question this point, until the bacterium has been obtained in a pure state.

*Cl. thermocellum* changes 70–80 per cent of the carbon of the cellulose decomposed into acetic acid (45 to 65 per cent), carbon dioxide, and smaller amounts of ethyl alcohol,  $\alpha$ -hydroxy acids, probably lactic, and residual acids, such as succinic. Glucose can be demonstrated as an end product of cellulose decomposition, frequently in considerable amounts.<sup>28</sup>

The organisms studied by Kroulik, Khouvine, Fred et al. produced no methane, while the culture obtained by Langwell and Lymn liberated methane in some cases. The quantitative relationship of the products formed by these organisms varied considerably. Coolhaas<sup>29</sup> expressed the opinion that the problem of cellulose decomposition under anaerobic conditions is still unsolved, due also to the fact that when pure cultures were obtained (including his own *Bac. thermocellulolyticus*), they could not decompose cellulose. He agrees with the earlier ideas of Keller-

<sup>28</sup> Scott, S. W., Fred, E. B. and Peterson, W. H. Jour. Ind. Engin. Chem. 22: 31. 1930.

<sup>29</sup> Coolhaas, C. Centrbl. Bakt. II, 75: 161–170, 344–360; 76: 38–44. 1928.

mann that cellulose fermentation or cellulose decomposition under anaerobic conditions is carried out by the combined action of an aerobic cellulose decomposing organism and an anaerobic acid and gas forming organism.

*Decomposition of cellulose by denitrifying bacteria.* Certain bacteria capable of reducing nitrates use cellulose as a source of energy. A medium containing 0.25 gram  $\text{KNO}_3$ , 0.05 gram  $\text{K}_2\text{HPO}_4$  and 2 grams of cellulose in the form of filter paper in 100 cc. of tap water is placed in a glass stoppered flask up to the neck, inoculated with canal slime and incubated anaerobically at  $35^\circ$ . Cellulose decomposition begins in a week and is accompanied by reduction of nitrate to nitrite. The nitrite also disappears in 15 days. On renewing the medium, the process of nitrate reduction is greatly hastened. The cellulose becomes orange yellow and of a slimy consistency. It is broken down into fibers which finally disappear. The gases consist of a mixture of nitrogen and carbon dioxide.

Groenewege<sup>30</sup> found that the process of cellulose decomposition in the presence of nitrates is carried out by the symbiotic action of two groups of organisms; one decomposes the cellulose, while the other reduces the nitrate using the products formed as a result of decomposition of the cellulose as a source of energy. By the symbiotic action of the two organisms the cellulose disappeared much more rapidly than by the action of the cellulose organism alone. Gas formation began to take place on the second day, accompanied by a reduction of nitrate to nitrite and NO with a gradual dissolution of the paper. The process was especially active when the culture solution was renewed by decantation.

To stimulate the development of cellulose-decomposing organisms, Van Iterson buried filter paper in the soil and after four weeks found that it became almost entirely decomposed and covered with orange and black spots. The black spots consisted of fungi. Using this material for inoculation, a complete reduction of 0.6 per cent  $\text{KNO}_3$  took place in three days. On replacing the solution by decantation, complete denitrification may take place in twenty-four hours. The organisms responsible for the process were obtained in pure cultures by the use of nutrient agar plates. A small piece of the decomposing cellulose is thoroughly disintegrated on the plate, by means of a sterile spatula. If a large piece of paper is used, it should be previously washed in salt solution, to wash off the rapidly growing organisms not taking part in the processes of cellulose decomposition and nitrate reduction. The specific bacteria develop only slowly as minute colonies; the plates have

<sup>30</sup> Groenewege, J. Bull. Jard. Bot. Buitenzorg. 2, f. 3: 261-345. 1920.

to be incubated for four to six days at room temperature, before transfers can be made. In the presence of rapidly growing organisms the minute colonies usually fail to develop.

The bacteria isolated in the processes of cellulose decomposition and nitrate reduction were divided<sup>30</sup> into three groups: (1) those which effect denitrification in nitrate bouillon but not in the cellulose-KNO<sub>3</sub> (0.1 per cent) medium; (2) those which do not reduce nitrates in the nitrate bouillon but cause reduction in the cellulose nitrate medium; (3) those which do not reduce nitrate in either medium. A combination of the first two groups or of all three brought about active reduction of nitrate and decomposition of cellulose. Group 1 was found to consist of two bacteria, namely *Bact. opalescens* and *Bact. viscosum*; group 2 consisted of three strains of *Bact. cellaresolvens* (a very fine, aerobic, rod-shaped organism), all of which grew very slowly and formed minute colonies on nutrient agar. The cellulose decomposing organism was obligate aerobic. *B. cellaresolvens* attacked cellulose; the products of the decomposition (acetic, butyric and lactic acids) could serve as food for the denitrifying organisms, *B. opalescens* and *B. viscosum*. The complete process is one of symbiosis.

For a quantitative study of cellulose decomposition, Groenewege used a medium consisting of:

Filter paper.....	200 mgm.	CaCO <sub>3</sub> .....	400 mgm.
NH <sub>4</sub> Cl.....	40 mgm.	Tap water.....	40 cc.
K <sub>2</sub> HPO <sub>4</sub> .....	20 mgm.		

The medium was inoculated with pure cultures of the organisms and mixtures of the cellulose decomposing and denitrifying forms. After three weeks, 10 cc. of 10 per cent HCl was added, flasks filled to half with water and after the cellulose settled, the liquid was taken off. This was repeated until the liquid was free from acid. The residue was then centrifuged, dried and weighed. The cellulose decomposing bacteria alone decomposed 17 to 135 mgm. of the cellulose (depending on strain) and 22 to 151 mgm. in the presence of the denitrifying bacteria. Similar results were obtained with asparagine as a source of nitrogen, indicating that the favorable influence of the *B. opalescens* on *B. cellaresolvens* is due to a symbiotic action.

Very little is known as yet concerning the specificity of hemicellulose-decomposing bacteria.<sup>30a</sup> The decomposition of one group of hemicelluloses, namely the pectins, has been studied most extensively.

*Pectin decomposing bacteria.* Pectins, like cellulose, are decomposed by (1) fungi, (2) aerobic bacteria, and (3) anaerobic bacteria.<sup>31</sup> The

<sup>30a</sup> Waksman, S. A. and Diehm, R. A. *Soil Sci.* **32**: 73-140. 1931.

<sup>31</sup> Hauman, L. *Ann. Inst. Past.* **16**: 379-385. 1902; Behrens, J. *Centrbl. Bakt.* **II**, 10: 524-530. 1903; Lafar's *Handb. techn. Mykol.* **3**: 269-286. 1904; Beijerinck, M. W. and van Delden, A. *Kon. Akad. Wetensch. te Amsterdam.* Dec. **19**: 673. 1903; Schardinger, F. *Centrbl. Bakt.* **II**, **22**: 98-103. 1909; Makrinov, I. A. *Arch. Sci. Biol.* **18**: No. 5. 1915; Rossi, G. and Guarneri, G. *R. Sc. Agr. Portici.* 1906; *Inter. Inst. Agr. Bur. Intel. Pl. Dis.* **7**: 635. 1916; Ruschmann, G. and Bavendamm, W. *Centrbl. Bakt.* **II**, **65**: 43-58. 1925; Jones, L. R. *N. Y. Agr. Exp. Sta. Tech. Bul.* **11**, 289-368. 1909.

aerobic bacteria capable of decomposing pectins include *Bac. asteroides* and *Bac. mesentericus*, *Bac. subtilis*, *Bact. fluorescens*, *Bac. macerans* and *Pectinobacter amylophilum*. *Bac. comesii* and *Bac. kramarii* found by Rossi to be active pectin-decomposing organisms are merely varieties of *Bac. mesentericus* or *Bac. asteroides*. Mention should also be made of the bacteria causing soft rots of vegetables, especially *Bac. carotovorus*, which are able to break down pectins readily.

The anaerobic bacteria capable of decomposing pectins include two organisms: (a) *Bac. amylobacter* and (b) *Bac. felsineus*. The former comprises a variety of forms described under different names, including *Plectridium*, *Clostridium* and *Granulobacter*.<sup>32</sup> However, not all forms of *Bac. amylobacter* are capable of retting flax. *Bac. felsineus* Carbone<sup>33</sup> is 3 to 5 by 0.3 to 0.4 $\mu$  and forms free spores 3 by 1.5 to 2 $\mu$  in size.

*Bacteria decomposing hydrocarbons and benzene ring compounds.* Petroleum, paraffin oil and other hydrocarbons can be readily used by a number of soil bacteria as sources of energy.<sup>34</sup>

To obtain an enriched culture, the following medium was employed:

Tap water.....	1000 cc.	CaCO <sub>3</sub> .....	trace
K <sub>2</sub> HPO <sub>4</sub> .....	0.5 gram	One of the	
NH <sub>4</sub> Cl.....	0.5 gram	paraffins.....	about 10.0 grams

Petroleum, benzine and paraffin oil can be used directly. The paraffin is first melted by warming and the solution is then vigorously shaken. The medium is inoculated with soil and incubated. At 20°, fluorescent and fat-splitting organisms develop; at 38°, *Mycobacteria* (4 to 10 by 0.5 to 1.5 $\mu$ ) and *Micr. paraffinae* develop. Söhngen found that 4 to 8 mgm. of petroleum were oxidized in 24 hours at 28°C. for every square centimeter of surface of solution. Pure cultures of the organisms were obtained on a medium consisting of:

Washed agar.....	20 grams	Distilled water.....	1000 cc.
K <sub>2</sub> HPO <sub>4</sub> .....	0.5 gram	Paraffin vapor.....	
MgSO <sub>4</sub> .....	0.5 gram		

The number of bacteria capable of oxidizing paraffin in the soil is very large, reaching 50,000 to 200,000 per gram of garden soil. Among the species isolated we find *Bact. fluorescens liquefaciens* and *non-liquefaciens*, *Bact. pyocyaneum*, *B. stutzeri*, *B. lipolyticum*  $\alpha$   $\beta$   $\gamma$  and  $\delta$ , *Micr. paraffinae*, and all fat-splitting forms. Tausz and Peter<sup>35</sup> isolated three organisms

<sup>32</sup> Winogradsky, S. Compt. Rend. Acad. Sci. 121: 742. 1895; Störmer, K. Mitt. deut. Landw. Gesell. 18: 193. 1903; Chem. Centrbl. 76: 41. 1905; Beijerinck and Van Delden, 1902 (p. 101).

<sup>33</sup> Carbone, D. and Tobler, F. Faserforsch. 2: 163-184. 1923.

<sup>34</sup> Söhngen, N. L. Centrbl. Bakt. II, 37: 595-609. 1913.

<sup>35</sup> Tausz, J. and Peter, M. Centrbl. Bakt. II, 49: 497-554. 1919.

from the soil capable of decomposing hydrocarbons: *Bact. aliphaticum*, *Bact. aliph. liquefaciens* and *Paraffinbacterium*. The first was grown on inorganic and organic media to which a few drops of *n*-hexan was added; it is 2 by 1.5 $\mu$  in size, motile by means of peritrichous flagella, Gram-negative and grows well with aliphatic hydrocarbons as the only source of carbon and energy. The second form was isolated on media containing naphthenes as cyclohexan and 1-3 dimethylcyclohexan. It is similar to the first, but is strongly proteolytic. The third organism was isolated on selective media consisting of inorganic solutions and paraffin oil; in liquid media, it forms motile rods, 4 to 6 by 2 $\mu$ , developing into long chains; rapid spore formation is characteristic and it is, therefore, related to *B. subtilis*.

Various bacteria oxidizing phenol to CO<sub>2</sub>, pyrocatechin to oxychinon, benzol to fatty acids and CO<sub>2</sub>, and decomposing toluol, xylol and guajacol were isolated from the soil.<sup>36</sup> These bacteria use aromatic compounds as sources of energy. They are found among various genera, namely *Micrococcus* (especially *M. sphaeroides*), *Mycoplana* and *Mycobacterium*, *Bacterium*, *Pseudomonas*, *Vibrio* and *Bacillus*.<sup>37</sup>

<sup>36</sup> Wagner, R. Ztschr. Gärungsphysiol. 4: 289-319. 1914.

<sup>37</sup> Gray, P. H. H. and Thornton, H. G. Centrbl. Bakt. II, 73: 74-96. 1928.

## CHAPTER IX

### SOIL ALGAE AND THEIR ACTIVITIES

*Introductory.* The microscopic chlorophyll-containing forms of the great plant division Thallophyta, the Algae, are represented in the soil by three large groups: the *Cyanophyceae*, *Chlorophyceae* and *Bacillariaceae*. The first contain, in addition to chlorophyll, also the pigments phycocyanin and carotin, and are, therefore, blue-green to violet or brown in color; the second usually contain only chlorophyll, but sometimes also xanthophyll, and are, therefore, grass-green or yellow-green; the third contain, in addition to chlorophyll, also carotin and xanthophyll, and are golden brown in color. The chlorophyll-bearing microscopic forms of life are also represented in the soil by the Euglenaceae and the Cryptomonadaceae (or Flagellata and Dinoflagellata), commonly classified with the Protozoa (Flagellata), and by the filamentous moss protonema, which belongs to the higher group of plants, the Bryophyta.

Algae and the autotrophic groups of bacteria are the only microorganisms in the soil that can synthesize organic matter from inorganic materials, the fungi and the heterotrophic bacteria depending for their energy upon organic matter synthesized by other forms of life. The autotrophic bacteria obtain their energy chemosynthetically, using inorganic substances as a source of energy; the algae obtain their energy photosynthetically, using the energy of the rays of the sun.

Algae are universally distributed on the surface of the soil, wherever moisture and light are available. It is sufficient to moisten the soil with water and expose it to the light to obtain in a short time an abundant vegetation. However, algae may also be living below the surface of the soil, not exposed to the direct rays of the sun and under more uniform temperature and moisture conditions. The algae, as well as the other groups of soil microorganisms grow in the soil in mixture and, for purposes of identification and particularly for physiological investigations, they have to be isolated and cultivated upon artificially prepared media.

Some of the algae are isolated readily from the soil and others only with difficulty. For morphological studies and classification, it is suffi-



cient to separate the various forms and to cultivate them under artificial conditions, even if they are contaminated with fungi or bacteria; but for physiological studies, especially the assimilation and transformation of various elements, as inorganic matter, decomposition, nitrogen fixation and symbiotic action, it is important to obtain them free from any contaminating organisms.

*Methods of isolation of impure cultures of algae.* The methods of isolation of algae from the soil fall into methods for obtaining enrichment, crude, pure and single-cell cultures. The enrichment culture methods consist in making conditions favorable for an abundant development of algae, for identification purposes; the pure culture methods deal with the processes of obtaining the organisms free not only from fungi and bacteria, but also from other algae, for physiological studies as well as for a more careful study of their morphological characteristics.<sup>1</sup> The enrichment culture is also the preliminary step in the isolation of the pure culture. Since algae require light for the autotrophic assimilation of carbon dioxide (photosynthetically), this need of light is utilized for enrichment purposes. A small quantity of soil is added to a large flask containing 0.02 per cent  $K_2HPO_4$  in tap water and is exposed to light; the development of various blue-green algae will soon take place. When dry soil is used as the inoculant, the spore-forming Nos-

<sup>1</sup> Wettstein, F. von. Österreich Bot. Ztg. 70: 23-28. 1921; Pringsheim, E. G. Beitr. Biol. Pfl. 14: 283-311. 1928.

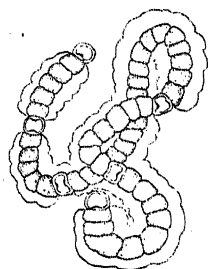
## PLATE IX

### SOIL ALGAE

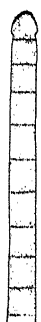
75. *Pleurococcus* sp. (from Robbins).
76. *Nostoc commune* (from Robbins).
77. *Microcoleus vaginatus* (from Robbins).
78. *Phormidium* sp. (from Robbins).
79. *Anabaena* sp. (from Robbins).
80. *Nodularia* sp. (from Robbins).
81. *Chlamydomonas communis*: 1-3, motile vegetative cells; 4, resting cell in which division is about to take place; 5-6, longitudinal fission into four zoogonidia; 7, oblique fission,  $\times 960$  (from Bristol).
82. *Ulothrix tenuissima*  $\times 550$  (from Bristol).
83. *Bumillaria exilis*: a and b, vegetative filaments showing variable number of chloroplasts,  $\times 550$ ; c and d, filaments showing stages in formation of zoogonidia,  $\times 960$  (from Bristol).
84. *Cylindrospermum muscicola*: a, typical filament in different stages of spore formation; b, spore formed in an irregular position,  $\times 550$  (from Bristol).
85. Some typical soil diatoms: 1-2, *Navicula borealis*; 3-5, *N. balfouriana*; 6-9, *N. intermedia*; 10-12, *N. brebissonii*, var. *diminuta*; 13, *N. elliptica*, var. *oblongella*; 14, *N. elliptica*, var. *minima*; 15-17, *N. terricola*,  $\times 960-1150$  (from Bristol).



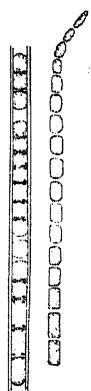
75



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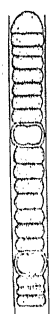
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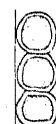
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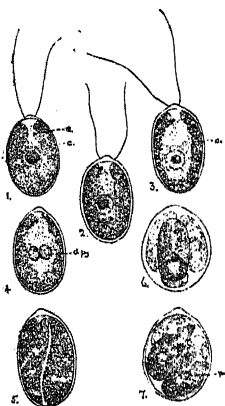
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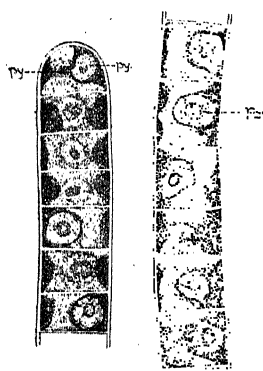
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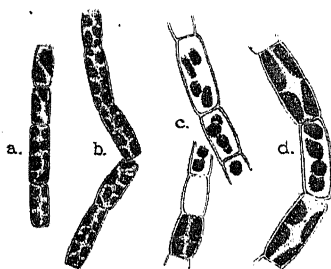
80



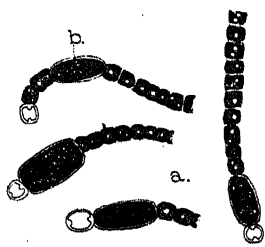
81



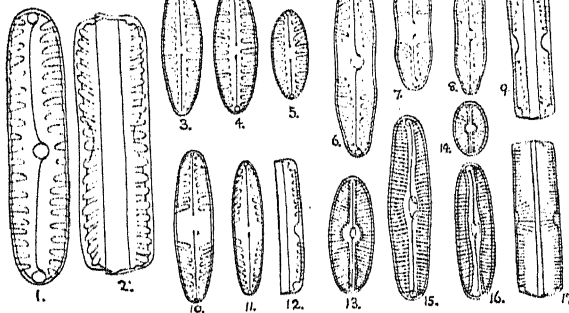
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85



tocaceae, such as *Anabaena* and *Cylindrospermum* will develop.<sup>2</sup> When a large quantity of soil is placed in tap water, various small diatoms will always develop. When proteins are added to the soil and covered with water, Volvocineae, such as *Chlamydomonas*, *Karteria*, *Chlorogonium*, *Spondylomorom* are obtained.<sup>3</sup>

For the purpose of isolating algae, Robbins<sup>4</sup> filled 500 cc. Florence flasks to their greatest diameter, with ground quartz, previously washed free from all suspended matter. The flasks are plugged with cotton and sterilized at 120°C. for 30 minutes. The soil inoculum is shaken, for 5 minutes, with sterile water; an amount (25 cc.) equivalent to 10 grams of soil is evenly distributed, with a sterile pipette, over the ground quartz surface. The flasks are tipped to one side so as to offer both a moist sand and a free water surface for the algae to grow on. The flasks are kept in the greenhouse in a sunny place, then in a shady place. After growth of algae has taken place, they are transferred to a 1 per cent agar medium with soil extract as a base. Some of the algal material is shaken in a test tube with a few cubic centimeters of sterile water, then transfers are made, with a platinum loop, to tubes of liquefied agar cooled to 42°C. The tubes are shaken and the agar is poured into sterile Petri dishes. Growth of algae will appear in 2 or 3 weeks. The cultures may then be transferred to insure purity. Moore and Karrer<sup>5</sup> placed about 1½ inches of sand in pint milk bottles, to which 150 cc. of a culture solution had been added. The bottles were plugged with cotton and sterilized at 8 to 10 pounds pressure for one-half hour.

The undiluted culture solution has the following composition:

NH <sub>4</sub> NO <sub>3</sub> .....	0.5 gram	CaCl <sub>2</sub> .....	0.1 gram
KH <sub>2</sub> PO <sub>4</sub> .....	0.2 gram	FeSO <sub>4</sub> .....	trace
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.2 gram	Distilled water.....	1000 cc.

The bottles are inoculated with about 10 grams of soil taken at the desired depth. To lessen the amount of evaporation, waxed paper covers are placed over the cotton plugs. The sand is slanted in the bottle so as not to be wholly submerged, giving various moisture conditions. The bottles are placed so as to get good light for at least part of the day. The water lost by evaporation is replaced from time to time with sterile water.

The following two media are also recommended for the isolation of algae:

<i>Bristol's solution</i> <sup>6</sup>		<i>Detmer's solution</i>	
NaNO <sub>3</sub> .....	0.5 gram	Ca(NO <sub>3</sub> ) <sub>2</sub> .....	1.0 gram
KH <sub>2</sub> PO <sub>4</sub> .....	0.5 gram	KH <sub>2</sub> PO <sub>4</sub> .....	0.25 gram
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.15 gram	KCl.....	0.25 gram
CaCl <sub>2</sub> .....	0.05 gram	MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.25 gram
NaCl.....	0.05 gram	Tap water.....	1000 cc.
FeCl <sub>3</sub> .....	0.005 gram		
Distilled water.....	1000 cc.		

<sup>2</sup> Beijerinck, M. W. Centrbl. Bakt. II, 7: 561-582. 1901.

<sup>3</sup> Jacobsen, H. C. Ztschr. Bot. 2: 145-188. 1910.

<sup>4</sup> Robbins, W. W. Colorado Agr. Exp. Sta. Bul. 184. 1912.

<sup>5</sup> Moore, G. T. and Karrer, J. L. Ann. Mo. Bot. Gard. 6: 281-307. 1919; Moore G. T., and Carter, N. Ibid. 13: 101-140. 1926; Jour. Appl. Microsc. 6: 2309-2314. 1903.

<sup>6</sup> Bristol, B. M. Ann. Bot. 34: 35-79. 1920.

Detmer's medium is diluted to one-third of its strength and 0.01 per cent  $\text{FeCl}_3$  is added. Distilled water is always prepared in a silver or glass still. Sand is placed into wide-mouth culture bottles to a depth of about 1.5 inches and moistened with one of the above media; the bottles are plugged and sterilized, then inoculated with a suspension of the soil in a sterile mineral salt solution.

The soil may also be packed in a Petri dish to a depth of about 1 cm., well moistened with sterile distilled water, and the surface covered with a piece of pure filter paper. The dishes are kept in diffuse light, preferably at a temperature of 20° to 25°C. The paper is moistened from time to time with sterile distilled water. After 2 to 60 days, various blue-green algae are found to grow through the pores of the paper to the light.<sup>7</sup> The mixed cultures are transferred to sterile nutrient solutions or proper agar media for the isolation of the individual species.

*Isolation of pure cultures.* The separation of various species of algae can be done either mechanically by the use of a loop and the microscope, or culturally by the use of solid media.<sup>8</sup> Beijerinck<sup>9</sup> was the first to isolate algae in pure culture, using a medium consisting of ditch water, to which 10 per cent gelatin had been added. The liquid media given above are suitable for the cultivation of algae. An alkaline reaction is most favorable, since algae are usually injured in acid media.

The agar plate method for the isolation of pure cultures of algae has been successfully employed.<sup>10</sup> The broken-up mass of algal material can be streaked out several times upon the surface of a solidified agar plate, so that each streak carries less of the inoculum than the preceding one. The inoculum may also be placed in a tube of melted and cooled agar; then a series of successive transfers are made into other tubes, so as to obtain a series of dilutions. These agar tubes inoculated with successively decreasing portions of material are poured into sterile Petri dishes. The streak method allows the development of surface colonies and the tube method of deep colonies. The plates are then exposed to light, so as to stimulate the development of the algae and to prevent the growth of other organisms. When the colonies have developed sufficiently they are transferred into liquid media. Algae are usually provided with a more or less highly developed exterior mucilaginous investment, either in the form of a

<sup>7</sup> Esmarch, F. *Hedwigia*, 55: 224-273. 1914; *Diss. Kiel*. 1914.

<sup>8</sup> Pringsheim, E. G. *Abderhald. Handb. biol. Arbeitsmeth.* Abt. XI, T. 2, 377-406. 1924.

<sup>9</sup> Beijerinck, M. W. *Bot. Ztg.* 48: 725-785. 1890; also *Centrbl. Bakt.*, 13: 368-373. 1893.

<sup>10</sup> Tischutkin, N. *Centrbl. Bakt.* II, 3: 183-188. 1897; Ward, H. M. *Ann. Bot.* 13: 563-566. 1899.

sheath or of a mere gelatinization. They also develop much more slowly than fungi. Both of these factors contribute to the difficulties encountered in pure culture work with algae.<sup>11</sup> When the life-history of the organism is known, the best period for obtaining it free from bacteria can be readily determined.

Schramm<sup>11</sup> used, for the isolation of algae, the medium recommended by Moore with the addition of 10 gm. of agar. The latter is carefully washed first in tap water, then in distilled water,<sup>12</sup> so that the medium can be cooled down to about 34.5° to 35°C., without solidification. Six to eight cubic centimeters of agar are placed in a Petri dish 10 cc. in diameter. If the alga is filamentous, it is first washed in sterilized nutrient solution; if it is a unicellular form, it is diluted, the extent of dilution depending on the abundance of organisms. The material is added to the tube of liquid agar, which is then vigorously shaken so as to separate the adhering bacteria, and the contents are poured into a Petri dish. The plates are allowed to cool, turned upside down, so as to prevent the moisture from spreading bacteria over the surface, and placed in the light of a north window, preferably in a glass case. The plates are examined frequently and, if rapidly spreading bacteria and fungi are found, they are dissected out. The algal colonies usually appear in from three to four weeks. If the inverted plates are examined from time to time with the compound microscope (12 m. objective) the algal colonies may be located in the very early stages of development. The colonies are marked with a glass pencil and are transferred, by means of a platinum loop, to sterile agar slants. The purity of the culture may be further tested by transferring it to media suitable for bacterial growth.

This method may have to be modified for particular forms of algae: in some instances the Barber pipette is used; in other cases the fact is utilized that certain species readily produce zoospores or other free endogenous spores; in some species the vegetative cells are either free from bacteria or can be rendered so by mechanical means. Pure cultures of various algae, particularly of Chlorophyceae, were thus isolated. The Cyanophyceae present more difficult problems of isolation, since the gelatinous investments are all impregnated with bacteria, which cannot be removed even by most vigorous washing. By the use of silicic acid gel, one species of *Oscillatoria* and one *Microcoleus* were isolated. However, as soon as these two organisms are completely separated from bacteria, the media, otherwise favorable, seem to become unfavorable and the organisms eventually die.

By repeated transfer to sterile silicic acid gel plates, a species of

<sup>11</sup> Schramm, J. R. Ann. Mo. Bot. Gard. 1: 23-45. 1914.

<sup>12</sup> Richter, O. Ber. deut. bot. Gesell. 21: 493-506. 1903; Monograph. Abhandl. Int. Rev. Hydrobiol. Hydrogr. 2: 31. 1911.

*Nostoc* was isolated<sup>13</sup> in pure culture. Another method<sup>14</sup> consists in growing the organisms in a dilute mineral salt solution, either placed in flasks or impregnated in silica gel. Subcultures are made for enrichment purposes. Dilute suspensions of the algae, well shaken for the separation of the cells, are inoculated into flasks containing melted and cooled (42°C.) agar that has been well shaken. The colonies are allowed to develop in the solid agar in bright sunlight, and are then cut out aseptically and transferred to fresh media. The process may have to be repeated.

*Cultivation of soil algae.* For the cultivation of soil algae, the above three solutions, either in liquid form or with 1.5 per cent agar, can be used. A medium containing 1.475 gram of  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , in place of 0.5 gram  $\text{NH}_4\text{NO}_3$ , per liter has been used<sup>15</sup> with good results. For the cultivation of diatoms, a modification of Miquel's medium, consisting of the following two solutions has been found<sup>16</sup> to give satisfactory results.

A. 20.2 grams  $\text{KNO}_3$  in 100 cc. of distilled water.

B. 4 grams  $\text{Na}_2\text{HPO}_4$  in 40 cc. water + 2 cc. concentrated  $\text{HCl}$  + 2 cc.  $\text{FeCl}_3$  (melted at 45°C.) + 4 grams  $\text{CaCl}_2$  dissolved in 40 cc. water.

Forty drops of A and 10 to 20 drops of B are added to 1 liter of distilled water.

In addition to the above media, the following were found to be very favorable for the growth of algae.<sup>17</sup>

<i>Pringsheim solution</i>		<i>Benecke solution</i>	
$\text{NH}_4\text{MgPO}_4$ .....	1.0 gram	$\text{Ca}(\text{NO}_3)_2$ .....	0.5 gram
$\text{K}_2\text{SO}_4$ .....	0.25 gram	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .....	0.1 gram
$\text{Fe}_2(\text{PO}_4)_2$ .....	trace	$\text{K}_2\text{HPO}_4$ .....	0.2 gram
Water.....	1000 cc.	$\text{FeCl}_3$ .....	trace
		Water.....	1000 cc.

<sup>13</sup> Pringsheim, E. G. Beitr. Biol. Pflanz. 11. 1912; 12: 49-108. 1913; Arch. Protistenk. 72: 1-48. 1930.

<sup>14</sup> Chodat, R. Étude critique et expérimentale sur le polymorphisme des algues. Genève. 1909; Monographie d'algues en culture pure. Matériaux pour la flore cryptogamique suisse. Vol. IV, Fasc. 2, 1913. Berne.

<sup>15</sup> Wann, F. B. Jour. Bot. 8: 1-29. 1921.

<sup>16</sup> Miquel, P. Le Diatomiste. 1: 93-99. 1890; Allen, E. J. and Nelson, E. W. Jour. Marine Biol. Assoc. 8: 421-474. 1907.

<sup>17</sup> Pringsheim, 1924 (p. 204); Benecke, W. Bot. Ztg. 56: 83-97. 1898; Chodat, R. and Grintzesco, J. Actes Congr. Intern. Bot. Paris. 1910, 157; Pringsheim, E. G. Arch. Protistenk. 72: 1-48. 1930.

*Chodat medium*

Ca(NO <sub>3</sub> ) <sub>2</sub> .....	1.0 gram	FeSO <sub>4</sub> .....	trace
K <sub>2</sub> HPO <sub>4</sub> .....	0.25 gram	Distilled water.....	1000 cc.
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.25 gram	Washed agar.....	10 grams
KCl.....	0.10 gram	pH.....	5.3-5.5

The KCl may be replaced by CaCl<sub>2</sub> and the reaction adjusted to pH 7.3.<sup>18</sup> Organic media may also be employed. Various proteins, including peptone, can be used as sources of nitrogen; sugars (glucose, fructose), higher alcohols (mannitol, glycerol), and organic acids (in the form of neutral salts) can be used as sources of carbon. Various infusions of hay, manure, peas and soil can also be used, especially when the N and P content of the latter is increased by the addition of inorganic salts. Of the various organic media suggested, mention may be made here of two<sup>19</sup>:

(a) Cane sugar.....	10 grams	(b) Malt extract.....	890 grams
Asparagine.....	2 grams	Glucose.....	29 grams
Peptone.....	8 grams	Peptone.....	0.5 gram
Gelatin.....	80 grams	Asparagine.....	0.5 gram
Water.....	900 cc.	Gelatin.....	80 grams

To obtain inorganic solid media, add 15 to 20 grams of washed agar to one of the above solutions. In addition, soil, peat, sand and gypsum blocks can be used very readily, the last two moistened with a nutrient solution. To eliminate all traces of organic matter silica gel can be employed.

*Occurrence and distribution of algae in the soil.* The soil is a favorable medium for the growth of algae, which require only a relatively small amount of moisture to replace that lost by the protoplast in drying.<sup>20</sup> In view of the fact that algae can develop on organic media also in the dark, their existence in the soil even below the surface is made possible.

The occurrence of algae in the soil, particularly that of diatoms, has been referred to by a number of earlier writers.<sup>21</sup> Esmarch attempted to determine the distribution of algae on the surface of the soil, their presence in the subsurface, and whether cultivation influenced

<sup>18</sup> Fred and Peterson, 1925 (p. 365).

<sup>19</sup> Beijerinck, M. W. Verh. Prov. Utrechtsch. Genootsch. Kunst en Wetensch. 1889, 35-52. (Centrbl. Bakt. 8: 460-462. 1890.)

<sup>20</sup> Fritsch, F. E., and Haines, F. M. Ann. Bot. 148: 683-728. 1923.

<sup>21</sup> Ehrenberg, 1836 (p. 89); Gregory, W. Amer. Jour. Sci. Arts, II, 21: 434-437. 1856; Esmarch, 1914 (p. 204); Hamburg. wiss. Anst. 28: 63-82. 1910.



their distribution. Four types of uncultivated soils were used: sandy heathland containing only traces of organic matter, marshy bog, forest humus, and moist sand. The various samples were often taken from quite different localities having the same type of soil. Only 3 out of 34 samples of the sandy heathland showed the presence of Cyanophyceae on the surface. Thirty-five samples of the marshy bog soil showed no blue-green algae, but contained a few diatoms and grass-green algae. Only 5 samples out of 40 of the forest humus soil contained blue green algae and only 5 species were obtained altogether from soils of this type. The moist sandy soils indicated numerous blue-green algae on the surface. Subsurface samples from below the uncultivated soils were destitute of algae except in the moist sandy soils where they were fairly extensive in distribution. A larger number of blue-green algae was found in cultivated soils. A clay soil, for example, contained 23 species of blue-green algae in 35 out of 37 samples and 29 out of 45 samples of sandy soil contained 12 different kinds of algae. In general, cultivated soils were found to contain a greater number of blue-green algae than uncultivated, possibly because of the difference in moisture and mineral content. Grass land was richer in species than arable land. Subsurface samples were obtained at a depth of 10 to 25 cm. and, in some cases, at 30 to 50 cm., in a manner to prevent surface contamination. Only a few of the samples, coming from soils where there were no surface forms, contained no blue-green algae. Eighteen separate species were isolated, the number of algae decreasing with depth. In all, 45 species were described, of which 34 belonged to the Oscillatoriaceae and Nostococaceae.

Esmarch ascribed the occurrence of subsurface forms to their being carried down by soil cultivation and by seepage of surface waters, as well as by earthworms and other soil organisms. By growing blue-green algae in the dark, or burying algae in the soil, then examining microscopically at various intervals of time, the filaments were found to become discolored, finally changing to a yellow color; the filaments disintegrated leaving only spores and heterocysts behind. On moistening and exposing these to light, blue-green growth again appeared. The conclusion was reached, therefore, that blue-green algae cannot persist beneath the surface for any length of time, because of the absence of light and the destructive influence of the soil itself.

Acid soils were reported<sup>22</sup> to contain a different algal flora from that

<sup>22</sup> Petersen, J. B. Danske Vidensk. Selsk. Skrifter. 7 Raekke, Naturv. og Mathem. Afd. 12: 7. 1915.

commonly found in alkaline or neutral soils. Twenty-four species and varietes of diatoms were found in field and garden soils, 5 in marshy soils, and comparatively few or none at all in forest and heath-land soils.

Robbins<sup>23</sup> sampled several Colorado soils which were very rich in nitrate, by removing first the loose débris on the surface, then taking samples from the upper 3 to 4 inches. Out of 21 different species recorded, there were 18 Cyanophyceae, 1 diatom, and only 1 unicellular organism belonging to the Chlorophyceae. The Nostocaceae were best represented. The most prevalent species were *Phormidium tenue*, *Nostoc* sp., *Anabaena* sp., *Nodularia harveyana*, and *Stigonema* sp.

A distinct subterranean algal flora independent of the nature of the soil and the locality was found by Moore and Karrer.<sup>24</sup> Some species multiplied even when buried at a depth of one meter. In view of the fact that these algae were found in compact undisturbed soil, the possibility suggested itself that algae are present in the soil in a vegetative condition and actually grow there and play a definite function in soil transformations.

The following list contains the algae found in the soil by Moore and Karrer and the greatest depth at which they occurred:

<i>Chlorococcum humicola</i> (Näg.) Rab.....	100 cm.
<i>Hantzschia amphioxys</i> (Ehr.) Grun.....	100 cm.
<i>Navicula atomoides</i> Grun.....	100 cm.
<i>Trochiscia</i> ?.....	80 cm.
<i>Stichococcus bacillaris</i> Næg.....	70 cm.
<i>Oscillatoria amphibia</i> Agardh.....	70 cm.
<i>Cladophora</i> sp.....	60 cm.
<i>Anabaena</i> sp.....	20 cm.
<i>Nitzschia kützingiana</i> Hilse.....	20 cm.
<i>Nostoc muscorum</i> Ag.....	20 cm.
<i>Oscillatoria chlorina</i> Kütz.....	20 cm.
<i>Oscillatoria subtilissima</i> Kütz.....	20 cm.
<i>Scytonema hofmanni</i> Ag.....	20 cm.
<i>Oscillatoria anoema</i> (Kütz) Gomont.....	surface
<i>Oscillatoria formosa</i> Bory.....	surface
<i>Oscillatoria splendida</i> Greville.....	surface

An extensive study of the algal flora of desiccated soils has been made by Bristol.<sup>25</sup> Forty-four samples of soil desiccated from 4 to 26 weeks

<sup>23</sup> Robbins, 1912 (p. 203).

<sup>24</sup> Moore and Karrer, 1919 (p. 203).

<sup>25</sup> Bristol, B. M. Ann. Bot. 34: 35-79. 1920; New. Phytol. 18: 3-4. 1919; 19: 92-107. 1920.

and from widely separated localities were examined; a widely distributed ecological plant formation consisting of moss protonema and algae was present in cultivated soils. In these soils, 64 species and varieties of algae consisting of 24 species of Cyanophyceae, 20 Chlorophyceae and 20 Bacillariales (diatoms) were found. The most important species in the plant-formation were *Hantzschia amphioxys*, *Trochiscia aspera*, *Chlorococcum humicola*, *Bumilleria exilis* and, to a less degree, *Ulothrix subtilis* var. *variabilis*; moss protonema was universally present. There seemed to be an association between three blue-greens; namely, *Phormidium tenue*, *Ph. autumnale* and *Plectonema battersii*, two of which were found together in 16 of the samples and all three in 7 samples. Soils rich in blue-greens contained only a few species of diatoms, and vice versa; the first occurred more frequently in arable soils and the second in old garden soils. The resting forms could survive desiccation for a long period of time, 9 species of blue-green algae, 4 grass-greens and 1 diatom were isolated from soils stored for about 40 years; the *Nostoc muscorum* and *Nodularia harveyana* retained vitality for the longest period of time.

Most of the algae, except a few diatoms, are severely affected by frost, so that their numbers and activities usually reach a minimum in February. As soon as the snow melts a rapid development takes place, followed again by a minimum growth in the late summer.<sup>26</sup> With an increase in moisture, there is an increase in the numbers and activities. However, this phenomenon is not absolute. Observations by West and others indicate that different species of fresh water algae attain their maximum growth at different periods in the year. Soil algae show a similar variability.<sup>27</sup>

A detailed summary of the occurrence of algae in different soils is given in Table 18. The abundance of different species of algae in soil varies with depth, as shown in Table 19.

*Biochemical activities of algae.*<sup>28</sup> The blue-green algae consist of a mass of protoplasm surrounded by a very thin membrane, without a

<sup>26</sup> Francé, 1921 (p. 565); Magdeburg, P. Hedwigia. 66: 1-26. 1926.

<sup>27</sup> Bristol Roach, B. M. Abderhald. Handb. Biochem. Arb. Meth. Abt. XI, T. 3. 1926; see Chodat, F. Act. Soc. Helv. Sci. Natur. Lausan. 2: 191-192. 1928.

<sup>28</sup> The following contributions should also be consulted on the carbon utilization of algae: Artari, A. Ber. deut. bot. Gesell. 19: 7. 1901; Jahrb. wiss. Bot. 52: 410-466. 1913; 53: 527-535. 1914; Chodat, 1913 (p. 206); Bokorny, Th. Hedwigia, 59: 340-393. 1918; Dangeard, P. A. Compt. Rend. Acad. Sci. 172: 254-260. 1921; Grintzesco, J. Rev. Gen. Bot. 15: 5-19, 67-82. 1903; Roberg, M.

cell wall and without a nucleus.<sup>29</sup> The blue-greens, as well as other algae, contain one or more pigments<sup>30</sup> including chlorophyll which enables them to utilize the energy of the rays of the sun. They synthesize their protoplasm from the CO<sub>2</sub> of the atmosphere and from water containing inorganic nitrogenous and mineral compounds. Some algae, however, can also utilize organic materials, the extent depending on the species; some may thus develop in the complete absence of light, leading a heterotrophic existence. Under those conditions, the chlorophyll may be either completely lost or retained. Some species can even utilize organic nitrogenous compounds, and may bring about decomposition of proteins (Chodat).

Algae prefer nitrates as a source of nitrogen; ammonium salts are less favorable. Of the nitrates, Ca(NO<sub>3</sub>)<sub>2</sub> is best, followed by KNO<sub>3</sub> and NaNO<sub>3</sub>. The secondary ammonium phosphate is preferable to the other salts of ammonium, for, when the ammonium is used up, the secondary phosphate will be changed to the primary, which is only slightly acid, but the sulfate and chloride will leave the reaction of the medium acid. The preferential utilization of certain nitrogen sources may be due to a large extent to secondary reactions brought about by the residual ions. As various species behave differently toward the different sources of nitrogen, until further work has been done with a large number of species, no general conclusions can be drawn. Nitrites are not favorable, but can be utilized under proper conditions of reaction and concentration. In addition to water, carbon dioxide and nitrogen source, algae require for nutrition K, Fe, Mg, P, S, and in most cases also Ca. These are best added in the form of potassium phosphate and magnesium sulfate; a trace of iron is added in the form of chloride; and calcium, as sulfate, if it is not used as nitrate. The salts are used only in very dilute solutions<sup>31</sup>: Ca(NO<sub>3</sub>)<sub>2</sub> as 0.1 per cent, MgSO<sub>4</sub> as 0.01 per cent and K<sub>2</sub>HPO<sub>4</sub> as 0.02 per cent. A faintly alkaline reaction, as given by secondary phosphate and alkali bicarbonate, is best.

A biochemical process believed to be carried out by algae, namely, their ability to fix atmospheric nitrogen attracted considerable attention. As most of the earlier work in this connection has been carried out with

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Jahrb. wiss. Bot. **72**: 369-384. 1930; Ternetz, C. Jahrb. Wiss. Bot. **51**: 435-514. 1912; Bristol Roach, B. M. Ann. Bot. **40**: 149-201. 1926; **42**: 317-345. 1928; Ann. Appl. Biol. **14**: 509-518. 1927.

<sup>29</sup> Prát, S. Arch. Protistenk. **52**: 142-165. 1925.

<sup>30</sup> Kylin, H. Ztschr. physiol. Chem. **166**: 39-77. 1927.

<sup>31</sup> Richter, 1911 (p. 205).



[illegible]

TABLE 18—Concluded

	ROBBINS	ESMARCH	BRIESTOL	MOORE ET AL.	MOORE AND CARTER	FRANCÉ	PETERSEN	MAGDER- BURG
	Colorado soils	North German soils	Desic- cated and normal English soils	American soils	Sub- surface American soils <sup>4</sup>	South German soils	Danish soils	German peat (sphag- num) soils
<b>CHLOROPHYCEAE—grass green algae<sup>3,4</sup></b>								
<i>Chlamydomonas</i> .....			*	.....	*	*	.....	.....
<i>Gloeococcus</i> .....				.....		*	.....	.....
<i>Gloeocystis</i> .....				.....		*	.....	.....
<i>Chlorococcum</i> .....			*	.....	*		.....	.....
<i>Chlorochytrium</i> .....			*	.....	*		.....	.....
<i>Pleurococcus</i> .....	*			.....		*	*	.....
<i>Coccomyxa</i> .....			*	.....			*	.....
<i>Dactylococcus</i> .....			*	.....	*		*	.....
<i>Chlorella</i> .....			*	.....	*	*	*	.....
<i>Scenedesmus</i> .....				.....		*	.....	.....
<i>Ankistrodesmus</i> .....			*	.....	*		.....	.....
<i>Trochiscia</i> .....			*	*			.....	.....
<i>Raphidium</i> .....				*		*	.....	.....
<i>Ulothrix</i> .....			*	*	*	*	*	.....
<i>Stichococcus</i> .....			*	*	*		.....	.....
<i>Gongrosira</i> .....				.....			.....	.....
<i>Protoderma</i> .....				*		*	.....	.....
<i>Microspora</i> .....						*	*	.....
<i>Vaucheria</i> .....			*	.....			.....	.....
<i>Cladophora</i> .....				*			.....	.....
<i>Protosiphon</i> .....					*		.....	.....
<i>Monocilia</i> .....					*		.....	.....

<b>CONJUGATAE</b>									
<i>Mesotaenium</i> .....	.....	.....	.....	.....	.....	.....	.....	*	*
<i>Desmidiūm</i> .....	.....	.....	.....	.....	.....	.....	.....	*	*
<i>Euastrum</i> .....	.....	.....	.....	.....	.....	.....	.....	*	*
<i>Pleurotaenium</i> .....	.....	.....	.....	.....	.....	.....	.....	*	*
<i>Cylindrocystis</i> .....	.....	.....	.....	.....	.....	.....	.....	*	*
<i>Penium</i> .....	.....	.....	.....	.....	.....	.....	.....	.....	.....
<i>Tetmemorus</i> .....	.....	.....	.....	.....	.....	.....	.....	.....	*
<i>Staurastrum</i> .....	.....	.....	.....	.....	.....	.....	.....	.....	*
<i>Hyalotheca</i> .....	.....	.....	.....	.....	.....	.....	.....	.....	*
<i>Mougeotia</i> .....	.....	.....	.....	.....	.....	.....	.....	.....	*
<i>Zygogonium</i> .....	.....	.....	.....	.....	.....	.....	.....	.....	*
<b>HETEROKONTAE</b>									
<i>Tribonema</i> .....	.....	.....	.....	*	.....	.....	.....	.....	.....
<i>Bumilleria</i> .....	.....	.....	.....	*	.....	.....	.....	.....	.....
<i>Botrydium</i> .....	.....	.....	.....	.....	.....	.....	.....	*	.....
<i>Botrydiopsis</i> .....	.....	.....	.....	.....	.....	.....	*	.....	.....

<sup>32</sup> For a classification of the blue-green algae, see Tilden, J. E. Minn. Bot. Survey. 1910; Trans. Amer. Microscop. Soc. 36: 179-266, 1917; Forti-Sylloge Myxophycearum; the general texts of de Toni, Engler and Prantl, and West; Collins, F. S. Tufts College Studies, 4: No. 8. 1918.

<sup>33</sup> For a classification of diatoms see vol. x by Schonfeldt of Pascher's series; van Heurck, Traité des Diatomées; Taylor, F. B. Notes on diatoms. W. Watson & Sons, London. 1929.

<sup>34</sup> For a classification of the grass-green algae see West, Pascher, De Toni and Collins. The Heterokontae are discussed in a recent paper by E. M. Poulton. New Phytol. 29: 27-43. 1930.



impure cultures contaminated with various bacteria, results obtained under these conditions were not reliable. The negative results could be depended upon more than the positive results. Frank<sup>35</sup> suggested in 1889 that algae are able to fix atmospheric nitrogen; his results were substantiated by other workers, who used impure cultures of algae. Kossowitch,<sup>36</sup> however, who was the first to use pure cultures of algae, namely a species of *Cystococcus* and *Chlorella vulgaris*, demonstrated that algae do not fix any atmospheric nitrogen, and that the bacteria are the only organisms capable of doing that. In general, whenever pure cultures of algae were employed, nitrogen fixation was found to be

TABLE 19

*Estimated numbers of common species of algae at different depths of some normal English soils<sup>37</sup>*

NAME OF ORGANISM	UNMANURED SOIL				MANURED SOIL			
	Depth in centimeters							
	Surface	5	10	15	Surface	5	10	15
<i>Chlorococcum humicola</i> .....	2,072	1,300	2,072	407	3,316	5,325	18,138	5,325
<i>Pleurococcus vulgaris</i> .....	141	648	162	80	3,316	514	648	181
<i>Chlorella</i> sp.....	11,000	5,325	4,196	1,030	23,752	14,171	23,752	1,642
<i>Bumilleria exilis</i> .....	204	37	407	49	8,626	648	648	257
<i>Heterococcus viridis</i> .....	1,030	817	5,325	1,642	18,138	5,325	4,196	5,325
<i>Protococcus viridis</i> .....	323	1,642	14,171	648	407	514	6,780	1,030
<i>Chlamydomonas muscicola</i> ...	648	648	2,072	407	3,316	323	648	101
<i>Stichococcus bacillaris</i> .....	181	5	37		80	162	37	
<i>Hantzschia amphioxys</i> .....	37				49	80	27	19
<i>Navicula atomus</i> .....	37			19	27	49	37	37

negative.<sup>38</sup> But even if algae do not fix any nitrogen by themselves, they were found to exert a favorable effect on the process of nitrogen-fixation by non-symbiotic bacteria.<sup>39</sup> They build up considerable quantities of starch,<sup>40</sup> which may possibly be used by the bacteria as a source of energy.

<sup>35</sup> Frank, B. Ber. deut. bot. Gesell, 7: 34-42. 1889; Landw. Jahrb. 17: 421-453. 1888.

<sup>36</sup> Kossowitsch, P. Bot. Ztg. 52: 97-116. 1894.

<sup>37</sup> Bristol Roach, B. M. Jour. Agr. Sci. 17: 563-588. 1928.

<sup>38</sup> Charpentier, P. G. Ann. Inst. Past. 17: 321-334, 369-420. 1903.

<sup>39</sup> Krüger, W. and Schneidewind, W. Landw. Jahrb. 29: 776-804. 1900; Nakano, H. Jour. Coll. Sci., Tokyo Univ. 40: 1-214. 1917; Pringsheim, 1913 (p. 206); Jones, J. Ann. Bot. 44: 721-740. 1930; see also p. 117.

<sup>40</sup> Zurda, V. Beitr. Bot. Centrbl. 45: 97-270. 1928.

Beijerinck considered those organisms that can live on media to which no nitrogen has been added (without being, however, free from combined nitrogen), to be able to fix atmospheric nitrogen; he concluded, therefore, that various Cyanophyceae are able to fix atmospheric nitrogen, since they grew in media almost free from combined nitrogen. These media were not free from nitrogen-fixing bacteria, however, and no analytical data were presented. In a series of carefully controlled experiments with pure cultures of several grass-green algae, Schramm<sup>41</sup> came to the conclusion that *Chlamydomonas pisiiformis* Dill. forma minor Spargo, *Protosiphon botryoides* (Kütz.) Klebs, *Chlorococcum humicola* (Nägeli) Rabenh., *Chlorella vulgaris* Beij., *Stichococcus bacillaris* Næg., *Chlorella* sp. and *Kirchneriella* sp. are unable to fix free atmospheric nitrogen in the complete absence of combined nitrogen. Wann,<sup>42</sup> however, working in Schramm's laboratory claimed to have found that seven species of algae exhibited the ability to fix atmospheric nitrogen, when grown in pure cultures on mineral nutrient agar media containing either ammonium nitrate or calcium nitrate, as a source of nitrogen, and a small amount of glucose. A gain of 1 to 12.5 mgm. of nitrogen per flask was obtained. In the absence of glucose, growth and nitrogen-fixation were only slight. When urea, glycocoll, asparagine or ammonium sulfate was supplied as a source of nitrogen, either with or without glucose or mannitol, no fixation took place; one species caused a loss of nitrogen. Positive nitrogen-fixation by algae has also been claimed by Moore and Webster.<sup>43</sup> The fact, however, that bacteria were present in the cultures and that these were exposed to the air, from which traces of ammonia could be absorbed, would tend to invalidate these results.

Bristol and Page,<sup>44</sup> in a series of carefully controlled experiments, repeated Wann's work, using four different species of algae, each growing on six different media selected from among those used by Wann. They found no evidence to indicate any fixation of atmospheric nitrogen. In the presence of combined nitrogen, good growth was obtained, but only the original nitrogen was recovered even where it had originally been present in the form of nitrate. Bristol and Page pointed out a

<sup>41</sup> Schramm, J. R. Ann. Mo. Bot. Gard. 1: 157-184. 1914.

<sup>42</sup> Wann, 1921 (p. 206).

<sup>43</sup> Moore, B. and Webster, T. A. Proc. Roy. Soc. B. 91: 201-215. 1920; also 92: 51-60. 1921.

<sup>44</sup> Bristol, B. M. and Page, H. J. Ann. Appl. Biol. 10: 378-408. 1923.

serious source of error in the chemical method used by Wann for the determination of the initial nitrogen content of media containing nitrates and suggested that his apparent fixation of nitrogen was probably the outcome of a faulty chemical technic since they completely failed to corroborate his results. They also suggested that the results of Moore and co-workers were of doubtful validity, since their cultures of algae were probably not free from bacteria. Bacteria can develop in the gelatinous sheaths of algae and need, therefore, not cause any turbidity of the medium.

More recent studies<sup>45</sup> seem to indicate the possibility that blue-green algae are capable of fixing nitrogen.

Algae possess the capacity to withstand prolonged drought, during which time very little respiration is occurring. When the amount of moisture is increased, respiration increases, until the water content becomes so high as to interfere with the exchange of gases. In the case of some algae, a small increase in the moisture content of the dry material results in an immediate increase in the rate of respiration. Some algae possess a great water-retaining capacity.<sup>46</sup>

*Rôle of algae in the soil.* It is impossible to generalize concerning the rôle that algae may play in soil processes. They seem to be able to fix nitrogen by living symbiotically with nitrogen-fixing bacteria. They may also accumulate organic matter in the soil, but since they need available nitrogen, they may compete with higher plants for the soluble minerals and available nitrogen compounds in the soil. Gautier and Drouin<sup>47</sup> exposed samples of artificial soil, free from organic material and containing only ammoniacal nitrogen, in a sheltered position for a considerable period of time; the soil became, in course of time, covered with algae (*Pleurococcus vulgaris*, *Protococcus viridis*, etc.); this resulted in a loss in total nitrogen, a still greater loss in ammonia nitrogen, and a gain in organic nitrogen; the ammonia nitrogen was converted into organic nitrogen by the algae; with an increase in growth, there was a decrease in the loss of the total nitrogen. The algae thus play also a part in preventing the loss of ammonia nitrogen, as well as the leaching out of nitrates from the soil. The probable rôle of algae may thus con-

<sup>45</sup> Drewes. Centrbl. Bakt. II, 76: 88-101. 1928; Allison, F. Proc. Second. Intern. Congr. Soil Sci. III Comm. 1930; Science, N. S. 71: 221-223. 1930.

<sup>46</sup> Fritsch, F. E. Ann. Bot. 36: 1-20. 1922; 37: 683-728. 1923; 42: 75-100. 1928; Howland, L. J. Ibid., 43: 173-202. 1929.

<sup>47</sup> Gautier, A. and Drouin, R. Compt. Rend. Acad. Sci. 106: 754-7, 863-6, 944-7, 1098-1101, 1174-6, 1232-4. 1888; 113: 820-825. 1891.

sist in accumulating organic matter in newly formed soils.<sup>48</sup> It has been suggested<sup>49</sup> that algae, by taking in CO<sub>2</sub> and giving off oxygen, make swamp soils suitable for the growth of the rice plant. The roots of rice plants are typical land roots and possess no special adaptations to growth under swamp conditions. The large supply of dissolved oxygen in the swamp water produced by the photosynthetic activity of the algae enables the rice plants to grow under these artificial conditions.<sup>50</sup>

The fact that algae are present in the soil in considerable numbers, that they can grow even in the subsoil and in the dark, that they retain their vitality for very long periods, even after prolonged drought, that they can store large quantities of energy thus making them available for other organisms, all point to their probable importance in the soil. Algae also exert a solvent action upon insoluble calcareous materials (Chodat). In this respect, algae together with certain autotrophic bacteria play an important rôle in the disintegration of rocks and in the formation of soils. The possible rôle of algae in the deposition of limonite has also been suggested.<sup>51</sup> Algae may play an important rôle in the formation of certain limestones<sup>52</sup> and in the building up of certain peat bogs.<sup>53</sup>

<sup>48</sup> Treub, M. *Ann. Jard. Buitenzorg*. 7: 213-223. 1888; Fritsch, F. E. *Geogr. Jour.* 30: 531-548. 1907.

<sup>49</sup> Harrison, W. H. and Aiyer, S. *Pusa Mem., Chem. Series*, 3: 65-106. 1913; 4: 1-17. 1914.

<sup>50</sup> Brizi, U. *Ann. dell. Instit. Agr. Dott. A. Buti*. 5: 79-95. 1904; 6: 61-103. 1905; 7: 104-174. 1908.

<sup>51</sup> Steinecke, F. *Bot. Archiv*, 4: 403-405. 1923.

<sup>52</sup> Glock, W. S. *Amer. Jour. Sci. (V)* 6: 377-408. 1923.

<sup>53</sup> Dachnowski-Stokes, A. P. and Allison, R. V. *Jour. Wash. Acad. Sci.* 18: 476-480. 1928.

## CHAPTER X

### SOIL FUNGI

*Occurrence of fungi in the soil.* Fungi are heterotrophic organisms and depend for their energy supply on the decomposition of plant and animal substances; their existence in the soil is thus closely connected with the decomposition of the organic matter added to the soil. In respect to their rôle in soil processes, fungi can be divided into two distinct groups: (1) those that live freely in the soil, largely the ordinary filamentous fungi or molds, and (2) those that are capable of forming mycorrhiza with higher plants. The higher or mushroom fungi are found both among the free living forms and among those forming mycorrhiza. In addition to these two general groups, the soil also harbors various fungi capable of causing plant diseases.

It has been known for some time that fungi occur abundantly in the soil, particularly in soils rich in organic matter and acid in reaction. But in view of the fact that fungi are present in the soil both in the form of vegetative mycelium and as reproductive spores, it is rather difficult to estimate their abundance; it is even less possible to find a basis for comparing the relative abundance of fungi and bacteria and their capacity for causing a certain amount of transformation in the soil. It has been recognized<sup>1</sup> that, under certain conditions, particularly in uncultivated soils and below the layer containing humus, fungi may be as abundant as, if not more so, than bacteria. The earlier workers<sup>2</sup> emphasized the fact that fungi predominate in acid soils, and bacteria in neutral soils. This is true only to a certain extent. Fungi can, as a rule, withstand greater concentrations of acidity than bacteria and actinomyces, so that, at a pH of 4.0, the soil may contain only small numbers of the last two groups or organisms, while the fungi may still be present in abundance. At less acid reactions, when conditions are favorable for cultivated plants (pH 4.6 to 6.5), bacteria occur most abundantly, whereas the numbers of fungi will depend on the soil reaction, the amount of organic matter, and the abundance of water in the soil.

Our knowledge of the fungi of the soil dates back to 1886, when the

<sup>1</sup> Moore, G. T. *Science*, **36**: 609-615. 1912.

<sup>2</sup> Ramann, E. *Bodenkunde*. Berlin, Springer. 1920.

first<sup>3</sup> attempts were made to isolate fungi from the soil and to give them names and descriptions. However, only in 1902, Oudemans and Koning<sup>4</sup> made an attempt at a systematic study of the occurrence of fungi in the soil and their proper classification. In 1908 appeared the work of Hagem<sup>5</sup> and Lendner<sup>6</sup> on the Mucorales of the soil. This was soon followed by other investigations<sup>7</sup> on the occurrence of fungi in various types of soil, under different climatic and other environmental conditions, and by contributions dealing with one or more groups of soil fungi. These were either limited to the isolation of a few forms for biochemical purposes, or they dealt with an important group of soil organisms like the Mucors,<sup>8</sup> or with representatives of various groups in the study of one important soil process, like nitrogen fixation<sup>9</sup> or cellulose decomposition.<sup>10</sup> A number of papers and monographs are available which are devoted to certain groups of fungi, some of which were not isolated from the soil although they are of common occurrence in the soil. These are of much assistance in the study and identification of the soil forms. Reference must be made here to the work of Wehmer, Thom<sup>11</sup> and others on the genus *Aspergillus*; of Thom, Westling, Sopp and Biourge<sup>12</sup> on *Penicillium*; of Hanzawa<sup>13</sup> on *Rhizopus*; of Butler<sup>14</sup>

<sup>3</sup> Adametz, L. Inaug. Diss. Leipzig, 1886.

<sup>4</sup> Oudemans, C. A. J. A. and Koning, C. J. Arch. Neerland. Sci. Exact. et Nat. (2), 7: 266-298. 1902.

<sup>5</sup> Hagem, O. Vidensk. Selsk., I Math. Naturw. Klasse, 7: 1-50. 1907; 10: 1-52. 1910; Ann. Mycol. 8: 265-286. 1910.

<sup>6</sup> Lendner, A. Les Mucorinées de la Suisse. Bern, 1908.

<sup>7</sup> Jensen, C. N. Cornell Univ. Agr. Exp. Sta. Bul. 315, 1912; Dale, E. Ann. Mycol. 10: 452-477. 1912; 12: 33-62. 1914; Waksman, S. A. Soil Sci. 2: 103-155. 1916; 3: 565-589. 1917; Gilman, J. C. and Abbott, E. V. Iowa St. Coll. Jour. Sci. 1: 225-345. 1927.

<sup>8</sup> Povah, A. H. W. Bull. Torrey. Bot. Club, 44: 241-259, 287-313. 1917.

<sup>9</sup> Goddard, 1913 (p. 242).

<sup>10</sup> Daszewska, 1913 (p. 248); Traaen, A. E. Nyt. Magaz. Naturw. Christiania, 52: 21-121. 1914.

<sup>11</sup> Wehmer, C. Die Pilzgattung *Aspergillus*. Genève. 1901; Lafar's Handb. Techn. Mykol. 4: 192-238. 1905-7; Thom, C. and Church, M. B. The *Aspergilli*. The Williams & Wilkins Co., Baltimore, Md. 1926; Blochwitz, A. Ann. Mycol. 27: 185-204, 205-240. 1929; 28: 241-268. 1930; Tamiya, H. and Morita, S. Bot. Mag. Tokyo 44: 1, 79, 135, 209, 251, 306. 1930.

<sup>12</sup> Thom, C. The *Penicillia*. Williams & Wilkins Co. 1929; Westling, R. Inaug. Dissert. Upsala; Ark. Bot., 11: 1-156. 1911; Sopp, J. O. Vidensk. Selskr. I. Mat. Naturv. Kl., No. 11, Kristiania. 1912; Biourge, Ph. La cellule. 33: 1st. fasc. Louvain. 1923.

<sup>13</sup> Hanzawa, J. Mycol. Centrbl., 1: 406-409. 1912; 5: 230. 1915; Yamamoto, Y. Jour. Fac. Agr. Hokkaido Imp. Univ. 28: 1-101, 103-327. 1930.

<sup>14</sup> Butler, E. J. Mem. Dept. Agr. India, Bot. Ser. 5, 1: 1-160. 1907.

on Pythium; of Chivers<sup>15</sup> on Chaetomium; of Sherbakoff and Wollenweber<sup>16</sup> on Fusarium; and of Berkhout<sup>17</sup> on Monilia and allied forms.

The composition of the fungus flora of the soil changes, both quantitatively and qualitatively, with the nature of the soil. Hagem, for example, has shown that cultivated soils have a distinctly different population of Mucorales than pine forest soils. The influence of reaction on the fungus population of the soil can be seen from the following example:

A soil receiving manure year after year, in addition to minerals (pH 5.5), had 79,000 fungi, as determined by the plate method; the same soil receiving lime in addition to manure (pH 6.7) had only 10,000 fungi per gram. The soil receiving no manure or fertilizer (pH 5.1), had 87,000 fungi; the same soil limed (pH 7.0) had only 16,000 fungi. The soil receiving ammonium sulfate and minerals (pH 4.2) had 129,000 fungi; the same soil limed (pH 5.2) had 32,000.

*Methods of demonstrating the occurrence and abundance of fungi in the soil.* The methods of studying the occurrence of fungi in the soil can be divided into two groups; (1) Those methods which demonstrate the presence of particular fungi in the soil, without any reference to the question whether these occur there only in the form of spores or also in the form of vegetative mycelium. (2) Those methods which tend to establish the active growth stages of fungi in the soil, in the form of vegetative mycelium. The first method is usually carried out as follows:

Soil samples are taken under aseptic conditions into sterile containers. There is greater danger of exposing the soil to air contamination, in the study of fungi than of bacteria. Various fungus spores are very abundant in the ordinary bacteriological laboratory, and because of the smaller number of fungi than bacteria in the soil (or cells developing into colonies) this error introduced will be greater in the case of fungi. The presence of dust fungi will lead also to misstatements in reference to the types of fungi present in the soil.

A given quantity of soil is diluted with a definite amount of sterile tap water, and stirred, to separate the spores and pieces of mycelium from the soil particles. One-cubic-centimeter portions of the desired dilutions are then plated out with agar of definite composition and the plates are allowed to incubate for 48 to 72 hours at 25° to 30°C. Ordinary bacteriological media can be used for this purpose but acid media, having a reaction of pH = 4.0, are preferable for this first step of the isolation of fungi. The acidity prevents the bacteria from developing and the fungi can be isolated free from bacterial contaminations. A lower dilution can be employed, than would be the case with media upon which bacteria are able to

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<sup>15</sup> Chivers, A. H. Mem. Torrey Bot. Club, **14**: 155-240. 1915.

<sup>16</sup> Sherbakoff, C. D. Cornell Univ. Agr. Exp. Sta. Mem., **6**: 85-270. 1915;  
Reinking, O. A. and Wollenweber, H. W. Philippine Jour. Sci. **32**: 103-253. 1927.

<sup>17</sup> Berkhout, C. M. Diss. Univ. Utrecht. 1923.

grow; this allows the development of greater numbers and, therefore, of a greater variety of fungi. The medium described above (p. 18) can be used for this purpose. Any other sugar medium well adapted for the growth of fungi, to which some citric acid is added (about 1 per cent) can be used.<sup>18</sup> For the isolation of yeasts, a medium containing saccharose and 1.2 to 1.5 per cent citric acid is often recommended. Lactic acid can also be used. When fruit extracts, like raisin or plum extracts, are used as a base for the medium, no acid is required, since the natural acidity of the fruit is sufficient to prevent the development of bacteria. After the organism has been isolated, it is often necessary to obtain a single spore culture; especially when the organism is wanted for the study of hereditary characteristics or for physiological investigations.

For the demonstration of fungi present in the soil in the form of vegetative mycelium, the direct inoculation method<sup>19</sup> and the direct microscopic method<sup>20</sup> are available. According to the first method, lumps of soil, about 1 cc. in diameter, are placed, with a sterile forceps, into a sterile plate containing 10 cc. of sterile solidified agar medium. The plates are allowed to incubate for 24 hours at 22°C. This period of time is not sufficient for spores of the majority of soil fungi to germinate and form a mycelium visible to the naked eye, whereas the organisms actually living in the soil and forming a mycelium develop at once from the soil, so that the mycelium becomes visible earlier. After 24 hours' incubation the mycelium is transferred from the plates into sterile slants of fresh agar medium, care being taken to isolate the mycelium, which has grown away from the soil. The organisms thus isolated can now be cultivated, purified if necessary, and identified.

According to the second method, a small crumb (10 mgm. or less) of soil is placed upon a microscopic slide and mixed with two or three drops of water. A drop of methylene blue solution (saturated aqueous or Loeffler solution) is then added by means of a glass rod, well mixed with the soil suspension and covered with a cover slip. The preparation appears blue to the naked eye. If too much stain has been added, it is diluted by a drop of water. Examination is made with a dry lens and a highpower eye piece. By this method, fungus filaments can be found in all the soils examined. Some soils contain only 4 to 5 filaments in a preparation (comprising 5 to 10 mgm. of soil), while others, especially soils rich in undecomposed organic matter, contain fungus mycelium in

<sup>18</sup> Piettre, M. and de Souza, G. *Compt. Rend. Soc. Biol.* **86**: 336-337, 338-340. 1922.

<sup>19</sup> Waksman, S. A. *Science, N. S.*, **44**: 320-322. 1916; *Soil Sci.*, **14**: 153-158. 1922.

<sup>20</sup> Conn, H. J. *Soil Sci.*, **14**: 149-152. 1922.



great abundance. McLennan<sup>21</sup> demonstrated, by drying the soil in a vacuum desiccator over calcium chloride, that a marked reduction in the number of fungus colonies is obtained when the soil is plated out after this treatment; this was believed to prove that the normal fungus population of the soil is present largely in the condition of mycelium.

The microscopic method, however, gives no means of identifying the particular species of fungi present in the soil as vegetative mycelium. This would be rather difficult, since the very nature of the growth of fungi in the soil and on culture media is different.<sup>22</sup> The first method is also not without fault, since some fungi, especially those forming a long mycelium, like the Mucorales, make a more extensive growth upon the plate, than other fungi. Not only are the morphological characters of the organisms different in the soil and on culture media, but they may vary with different media. The same is true of course of the physiological activities of the fungi; freshly isolated organisms behave differently from those kept in culture on artificial media; young cultures from spores act differently from fully developed mycelium.

*Methods of cultivation of soil fungi.*<sup>23</sup> Fungi are cultivated to facilitate the study of their morphology, their reaction to environmental conditions, and their general physiology. The organisms, therefore, must be first isolated from the plate and grown in pure culture. This can be accomplished much more readily than in the case of algae or bacteria, since fungi grow rapidly, are largely aerobic, produce aerial spores abundantly, and can withstand comparatively large concentrations of acid.

The media for the cultivation of fungi may be designated as natural and artificial. Among the natural media, solid substrates including soil, hay, manure, fruits, bread, and branches are largely used for the growth of fungi. These are either kept at an optimum moisture or are previously sterilized, then inoculated with the organism. In the case of acid media, heating at 100° for 20 minutes may be sufficient for purposes of sterilization, but in the case of soil or hay, 2 hours at 15 pounds pressure or 30 minutes at 100° on seven consecutive days is required. Fruit

<sup>21</sup> McLennan, E. *Ann. Appl. Biol.* 15: 95-109. 1928.

<sup>22</sup> Church, M. B. and Thom, C. *Science, N. S.*, 54: 470-471. 1921.

<sup>23</sup> The cultivation of fungi is described in detail by O. Brefeld—*Untersuchungen auf dem Gesamtgebiete der Mykologie*, H. 14: 60. 1908; Küster, E. *Kultur der Microorganismen*. 3d Ed., 1921; Lafar's *Handb. d. tech. Mykol.* 1, 1904-1907; E. G. Pringsheim. *Abder. Handb. Biochem. Arb. Meth.* Abt. XI, T. 2: 407-444. 1921; E. Pribram. *Ibid.* XII, H. 3: 461-482. 1924.

extracts, as well as manure extracts, can also be used as nutrient solutions.

In the preparation of artificial media a nitrogen source, a carbon source, and minerals must be provided. Ammonium salts, nitrates and organic nitrogen compounds can be used as sources of nitrogen.<sup>24</sup> The ammonium salts can be used in the form of phosphate, sulfate, chloride and salts of organic acids, like acetic, tartaric and citric, in concentrations of 0.1 to 0.5 per cent. Ca, K, and Na nitrates can be used by almost all Aspergillaceae and various other fungi.<sup>25</sup> Among the organic nitrogenous compounds, peptone and amino acids (asparagine, leucine, etc.), followed by amides, amines, and alkaloids,<sup>26</sup> are found to be favorable sources of nitrogen.

Carbohydrates and higher alcohols are the best sources of carbon; of these, glucose comes first, followed by other hexoses and pentoses.<sup>27</sup> Sucrose is utilized only by fungi which can produce invertase; when added to an acid medium, it is inverted in the process of heating. Starch is utilized only by fungi which can produce diastase. It is employed either in the form of a paste or as soluble starch. Pectins are also used as sources of energy by various fungi.<sup>28</sup> Celluloses can be decomposed by certain fungi, hence mold activity is of great importance in the decomposition of organic matter in the soil. Of the alcohols, glycerol and mannitol are used most readily; the lower alcohols only in dilute solutions. Of the organic acids, those having more carbon atoms, like tartaric, citric, and malic, are best. Some fungi can utilize fats as sources of energy.<sup>29</sup>

Of the mineral elements, K, Mg, S, and P are necessary and cannot be replaced by others. If required Ca, Na, Cl need be present only in traces; Fe as well is sufficient in mere traces, when needed. Cu, Zn and Fe can act as stimulants.

<sup>24</sup> Brenner, W. *Centrbl. Bakt.*, II, 40: 555-647. 1914.

<sup>25</sup> Blochwitz, A. *Centrbl. Bakt.* II, 39: 499-502. 1913; Kossowicz. *Biochem. Ztschr.*, 67: 400. 1914.

<sup>26</sup> Ehrlich, F. *Mitt. landw. Inst. Breslau*, 6: 705-713. 1912; (*Centrbl. Bakt.* II, 41: 245-246. 1914).

<sup>27</sup> Peterson, W. H., Fred, E. B. and Schmidt, E. G. *Jour. Biol. Chem.*, 55: 19-34. 1922.

<sup>28</sup> Hauman, 1902 (p. 198); Behrens, 1903 (p. 198).

<sup>29</sup> Spieckermann, A. *Lafar's Handb. techn. Mykol.*, 2: 361-388. 1907; *Ztschr. Unters. Nahr. Genuszm.*, 27: 83. 1914; Rahn, O. *Centrbl. Bakt.* II, 15: 422-429. 1906 (see also p. 403).

The following media can be used for the cultivation of the great majority of soil fungi:

Czapek's solution consisting of:

Distilled water.....	1000 cc.	MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.5 gram
Cane sugar.....	30.0 grams	KCl.....	0.5 gram
NaNO <sub>3</sub> .....	2.0 grams	FeSO <sub>4</sub> .....	0.01 gram
K <sub>2</sub> HPO <sub>4</sub> .....	1.0 gram	(Sterilized at 15 pounds for 15 minutes)	

Since this medium contains cane sugar as a source of energy, and this is unfavorable for the development of the majority of Mucorales, another medium containing glucose as a source of energy should be employed for the isolation and cultivation of these forms. This medium has the following composition:

Glucose-peptone medium:<sup>30</sup>

Distilled water.....	1000 cc.	MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.25 gram
Peptone.....	10.0 grams	K <sub>2</sub> HPO <sub>4</sub> .....	0.25 gram
Glucose.....	20.0 grams	Agar.....	15.0 grams

Povah<sup>31</sup> employed the following medium for the isolation of Mucors:

NH <sub>4</sub> NO <sub>3</sub> .....	1.0 gram	Cane sugar..	5.0 grams
K <sub>2</sub> HPO <sub>4</sub> .....	0.5 gram	Agar.....	13.0 grams per liter
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.25 gram		

To prepare solid media, 10 or more per cent of gelatin or 1.5 to 2.0 per cent of agar is added to the above solutions. In the case of acid media (at pH 4.0), 3 per cent agar is required. In addition to the media mentioned above, Povah used another medium for stock cultures of Mucors:

Peptone.....	1.0 gram	Dry malt ex-	
Glucose.....	20.0 grams	tract.....	20.0 grams
		Agar.....	20.0 grams per liter

For the cultivation of the wood-destroying Basidiomycete, *Merulius lacrymans*, the following medium may be used:<sup>32</sup>

NH <sub>4</sub> NO <sub>3</sub> .....	10.0 grams	Lactic acid.....	2.0 grams
K <sub>2</sub> HPO <sub>4</sub> .....	5.0 grams	Water.....	1000 grams
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	1.0 gram		

50 cc. of this solution is added to 10 gm. of filter paper, the latter being used as a source of energy. Species of *Coprinus* can be cultivated upon sterile horse manure or manure decoction agar. *Agaricus* may be grown upon bread or bread mixed with sawdust.

Fungi will tolerate rather high concentrations of nutrients. *Asp. niger* has its optimum at 20 to 30 per cent cane sugar, its maximum in a solution containing 53 per cent glucose. The limiting osmotic pressure, when salts are used, is 17 to 21 atmospheres for *Asp. niger*

<sup>30</sup> Cook, M. T. Del. Agr. Exp. Sta. Bul. 91, 1911.

<sup>31</sup> Povah, 1917 (p. 221).

<sup>32</sup> Tubeuf, C. V. Centrbl. Bakt. II, 9: 127-135. 1902; Wehmer, C. Jahresh. Ver. Angew. Bot., 8: 178-198. 1911.

and for certain green *Penicillia*.<sup>33</sup> Fungi grow readily, in pure culture, at a wide range of reaction (see p. 245) and are not injured by high acidity as readily as bacteria; acid reactions, including acid soils, will, therefore, favor the development of fungi, in crude culture. With carbohydrates as sources of energy, the reaction of the medium becomes acid as a result of the growth of many fungi. With proteins and nitrates, however, the reaction will tend to become alkaline.<sup>34</sup> Fungi, with the exception of certain yeasts and certain Mucorales and Dematiaceae, are strictly aerobic; certain Mucors are capable of developing anaerobically, especially in the presence of available carbohydrates. Aeration will greatly stimulate the activities of most fungi, because of their strict aerobiosis.<sup>35</sup> The optimum temperature lies at 20 to 30° for the majority of species, in some cases (*Asp. niger*, *Mucor pusillus*) going up to 37°. Some fungi (*Penicillium expansum*, *Botrytis cinerea*, *Alternaria* sp.) germinate slowly at 0°, others (*Fusarium radicicola*, *Cephalothecium roseum*) germinate at 5°, whereas *Aspergillus niger* will germinate only above 10°. <sup>36</sup> In the case of certain fungi, like *Pen. expansum*, once growth has started at ordinary temperatures, the mycelium will continue to develop at 0°. Oxygen pressure has little effect upon the germination and growth of various fungi. Increased carbon dioxide pressure has a retarding effect, especially at low temperatures.<sup>35</sup> Heat has a destructive effect upon fungi; the spores of *Botrytis cinerea* are destroyed in ten minutes at 50.3°; the spores are, however, rather resistant to the action of sunlight.<sup>37</sup> Heating for thirty minutes at 62.8°C. is sufficient to destroy the conidia of most fungi, except certain species of *Aspergillus*.<sup>38</sup> The morphology of the fungi is appreciably affected by the composition of the medium. The nature of the mycelium, the rapidity of spore formation and the color of the culture will often depend, to a greater or less extent, upon the different constituents of the medium, its concentration, and the environmental conditions, such as temperature or aeration. For this reason, synthetic media and standard conditions

<sup>33</sup> Pringsheim, E. G. Ztschr. Bot., 6: 577-624. 1914; Haenseler, C. M. Amer. Jour. Bot. 8: 147-163. 1921.

<sup>34</sup> Bach, M. Compt. Rend. Acad. Sci., 178: 520-522. 1924.

<sup>35</sup> Kostytschew, S. and Afanassiewa, M. Jahrb. Wiss. Bot., 60: 628-650. 1921.

<sup>36</sup> Brooks, S. and Cooley, J. S. Jour. Agr. Res. 8: 139-164. 1917; Brown, W. Ann. Bot., 36: 257-283. 1922; Tomkins, R. J. Proc. Roy. Soc. 105B: 375-401. 1929.

<sup>37</sup> Smith, J. H. Ann. Appl. Biol., 10: 335-347. 1923; Weinzirl, J. Wis. Studies in Science. 1921, No. 2, 55-59.

<sup>38</sup> Thom, C. T. and Ayers, S. H. Jour. Agr. Res., 6: 153-166. 1916.

should be used in the study of the morphology and classification of such fungi as will thrive upon them.

*Isolation of single spore cultures.* In the study of fungi, especially their physiology, pure cultures from a single spore are prerequisites for any investigation. This was pointed out by Hagem and others. Some spore material is transferred by means of a platinum needle, to a flask containing about 30 cc. of sterile water. After vigorous shaking to separate the spores, a few cubic centimeters of the suspension is poured into a second flask containing sterile water. This is repeated once more, and 2 cc. of the final dilution is poured into a Petri dish containing solid nutrient material, moistening the whole surface of the plate; the excess water is then poured off. The plates are allowed to incubate for 2 to 3 days and are examined under the microscope for isolated growth derived from a single spore. This examination can be carried out by removing the cover from the Petri dish; also by examining the inverted dish under the microscope, using the low power and marking with a colored pencil the spot where an individual spore has germinated. If such a growth is found, it is transferred with a small amount of substrate, by means of a sterile platinum loop, to a new dish or sterile agar slant. Povah sprayed a spore dilution upon a poured plate by means of capillary pipettes, then proceeded as before. Within twenty-four hours, after the spore was removed, transfers were made from the edge of the growth to a fresh tube; the possibility of contamination through a neighboring spore delayed in germination was thus avoided.

The following procedure may also be followed:

## PLATE X

### SOIL FUNGI—MUCORALES

86. *Absidia glauca*, showing the runners with sporangiophores,  $\times 3.5$  (from Hagem).

87. *Absidia glauca*, columellae,  $\times 200$  (from Hagem).

88. *Rhizopus nigricans*, showing sporangiophores, rhizoids, and columellae,  $\times 40$ ; spores  $\times 360$  (from Jensen).

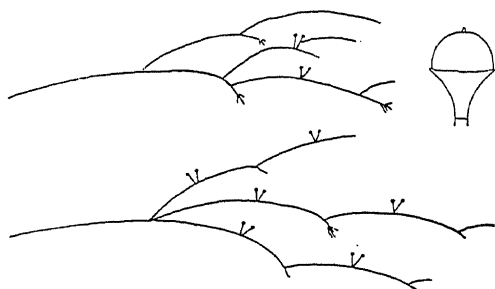
89. *Rhizopus arrhizus*: 1, runners with sporangiophores,  $\times 35$ ; 2, swollen sporangiophore,  $\times 35$ ; 3, abnormally divided sporangiophores,  $\times 35$ ; 4, columellae,  $\times 120$ ; 5, spores,  $\times 660$  (from Hagem).

90. *Zygorhynchus mölleri*, showing zygosporangium formation,  $\times 200$  (from Hagem).

91. Different forms of branching of *Mucors* (from Lendner).

92. Different forms of columellae of *Mucors*: a, spherical; b, spherical with persisting collarette; c, oval; d, oval depressed; e, pyriform; f, panduriform; g, conical; h, cylindro-conical; i, manniform; k and l, spinescent (from Lendner).

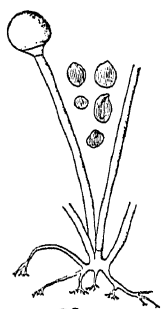
93. *Cunninghamella echinulata* (from Lendner).



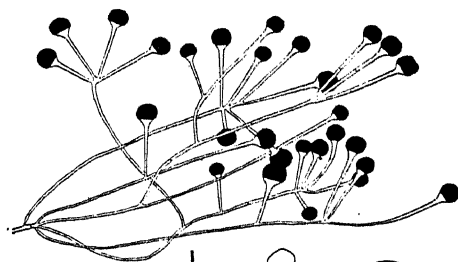
86



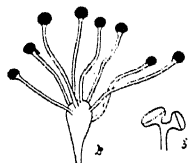
87



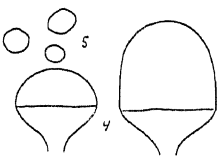
88



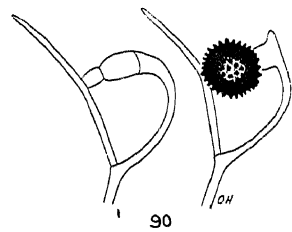
1



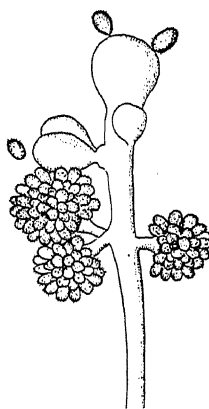
5



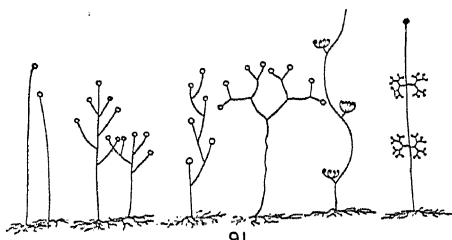
89



90



93



91



a



b



c



d



e



f



g



h



92



j



k



A small amount of the spore material is well shaken in 50 to 100 cc. of sterile water in an Erlenmeyer flask; a sterile platinum loop is then dipped into this suspension and carefully streaked out 3 or 4 times over the solidified agar in a Petri dish. The spores drop heavily at first, then singly, so that the third or fourth streak will have only single spores separated from one another. The plate is incubated for 36 to 48 hours, then examined in an inverted condition with the low power of the microscope. The streaks make easier the location of the spores. Where single spores have been dropped, they can be readily recognized, marked, and transferred with small blocks of agar into fresh dishes with agar or slants. By transferring again, within 24 hours, from the edge of the colony, and repeating the whole process when spore development takes place, single spore cultures can be assured. Of course, when the Barber pipette can be employed, there is greater certainty of obtaining single spore cultures. This is, however, not so necessary, in the case of fungi, as in the case of bacteria.<sup>39</sup>

Blakeslee<sup>40</sup> devised a procedure for the isolation of plus and minus strains of Mucorineae. This consists in teasing out an immature zygospore and placing it in a nutrient medium favorable for growth. In some cases, growth occurs from both suspensors in sufficient amount so that they can both be transferred to a fresh culture.

#### CLASSIFICATION OF FUNGI, WITH SPECIAL REFERENCE TO THOSE OCCURRING IN THE SOIL

**MYXOMYCETES.** The myxomycetes or slime-molds are characterized by the formation of naked masses of protoplasm or plasmodia, as a result of fusion of separate individuals. The plasmodium gives rise to spherical spores which in their turn produce on germination motile swarm cells. The myxomycetes were originally largely wood-inhabiting, but later adapted themselves to other substrates. The semi-fluid, easily motile vegetative body of the plasmodium is capable of attacking rapidly a fallen tree and utilize all the available nutrients. Coniferous woods are preferred to deciduous. The swarm cells are capable of ingesting spores of various fungi, there being a certain specificity in regard to the nature of the spores.<sup>41</sup>

The available information concerning the occurrence and activities of myxomycetes in the soil is very limited. The club-root of cabbage and other cruciferae (*Plasmodiophora brassicae*) once introduced into the soil will persist there for a considerable period of time.

At the present time 7 groups of myxomycetes are recognized<sup>42</sup>: 1. *Enteridiales*,

<sup>39</sup> Roberts, J. W. *Phytopathol.*, **13**: 558-560. 1923.

<sup>40</sup> Blakeslee, A. F. *Proc. Amer. Acad. Arts. Sci.*, **40**: 205-315. 1904.

<sup>41</sup> Gilbert, F. A. *Amer. Jour. Bot.* **15**: 473-484. 1928.

<sup>42</sup> John, E. *Ber. deut. bot. Gesell.* **46**: 8-17. 1928; Lister, G. A monograph of the mycetozoa. 3d Ed. British Museum, 1925; Macbride, Th. H. *North American slime-molds*. 2d Ed. Macmillan, New York. 1922; Wilson, M. and Cadman, E. J. *Trans. Roy. Soc. Edinburgh*. 1928; Brandza, M. *Bull. Soc. Mycol. France*, **44**: 249-299. 1929.



the plasmodium being often rose red. 2. *Cribrariales*, with dark grains in the plasmodium. 3. *Stemonitales*, the spores being brownish to violet-brown. 4. *Physarales* with violet-brown spores. 5. *Liceales*. 6. *Margaritales*. 7. *Trichiales*. Most of the species are cosmopolitan in nature. Brierley listed five genera of Myxomycetes as occurring in soil and probably leading there a vegetative existence. Thom and Raper found these organisms to constitute an active component of the microbial population of the soil and of the decomposing vegetation.<sup>42a</sup>

**EUMYCETES.** Vegetative tissues free from chlorophyll, unicellular or multicellular, with a typical apical growth and formation of ramified mycelium. Reproduction sexual and asexual. No locomotion in developed thallus.

**A. PHYCOMYCETES**<sup>43</sup> (algal fungi). Mycelium unicellular, unseptated, branched profusely, sexual reproduction by zygospore or oospore.

**I. ARCHIMYCETES.** Mycelium absent or rudimentary. Mostly parasitic. Some, like *Synchytrium endobioticum*, the cause of wart disease in potato, may persist in the soil for a long time. Some are parasitic upon soil fungi.

**II. OOMYCETES.** Sexual reproduction by oospores. Accessory spores often motile. Conjugating cells differing in appearance and function and consisting of a large oogonium and small antheridium.

1. **SAPROLEGNIALES**, unicellular, abundantly branched vegetative mycelium, asexual reproduction by means of conidia or swarm spores, produced in separate sporangia; sexual reproduction by means of an oogonium. Although Saprolegniales are commonly recognized as aquatic fungi, they have also been found extensively in the soil, some of them actually being more common in the soil than in water.<sup>44</sup> They are represented in the soil by a number of genera and species. The genus *Achlya* was found to be represented by 16 species, *Aphanomyces*—4 species (*A. laevis*, etc.), *Aplanes*, *Brevilegnia*—7 species, *Calyptralegnia*, *Dictyuchus*, *Geolegnia*, *Isoachlya*, *Leptolegnia*, *Pythiopsis*, *Saprolegnis*—4 species, as well as *Thraustotheca* and *Allomyces*.

2. **PERONOSPORALES** (downy mildews). This group is represented in the soil largely by the *Pythiaceae*, in which the sporangiophores differ little from the vegetative hyphae. The organisms lead in the soil a saprophytic existence, although many of them are facultative parasites. This is true of various species of *Pythium* represented in the soil by *P. de Baryanum*, the cause of the damping off disease of seedlings, *P. ultimum*, etc.; *Phytophthora* (*P. infestans*, etc.)

<sup>42a</sup> Thom, C. and Raper, K. B. Jour. Wash. Acad. Sci. 20: 362-370. 1930.

<sup>43</sup> Fischer, A. Rabenhorst's Kryptogamen Flora. I, 4: 1, 5. 1892; Lendner, 1908 (p. 221); Hagem, 1908 (p. 221).

<sup>44</sup> Harvey, J. V. Jour. Elisha Mitchell Sci. Soc. 41: 151-164; Coker, W. C. and Braxton, H. H. Ibid. 42: 139-149. 1926; Coker, W. C. Ibid. 42: 207-226. 1927; Couch, J. N. Ibid. 42: 227-242. 1927; Busse, W., Peters, L. and Ulrich, P. Arb. K. biol. Anst. Land. Forst. 8: 260-302. 1911.

III. ZYGOMYCETES: Sexual reproduction by fusion of terminal cells of branches of mycelium similar in appearance but different in sex. The most important group is the order MUCORALES. The soil is the primary source of this group of organisms, harboring as many as one-third of all known species.<sup>45</sup>

1. Reproduction asexually by spores contained in sporangia. Sub-order SPORANGIOPHORAE:

1'. Sporangia generally only of one kind, spherical or pyriform with a membrane that dissolves or fractures easily. MUCORACEAE.

1''. Sporangiphores arising from stolons (runners):

(a) Sporangiphores produced from the nodes of the stolons; spores often striated longitudinally; sporangia globose. *Rhizopus*. This genus is represented in the soil by a number of species, some of which, like *Rh. nigricans* (No. 88, Pl. X), *Rh. nodosus* and *Rh. arrhizus* (No. 89, Pl. X), have been isolated in different parts of the world by Hagem, Lendner, Dale and others.

(b) Sporangiphores produced from the internode of the stolon, sporangia pyriform. *Absidia* (Nos. 86-87, Pl. X). This genus is fairly well represented in the soil, although not very common; 12 species of *Absidia* have been described by Lendner. A number of species have been isolated by Hagem (*A. orchidis*, *A. glauca*, *A. spinosa*). Other species have been isolated by Oudemans and Koning, Dale, Waksman and others.

2''. No stolons are formed by the mycelium.

(a) Heterothallic, occasionally homothallic, but, in the latter case, the zygophores generally arise from comparatively distant parts of the mycelium, never formed between branches of a single aerial hypha, and are usually equal. *Mucor* (Nos. 91-92, Pl. X). This genus is one of the most common in the soil and is the largest in the number of species found in the soil. It has been studied in detail by Lendner, Hagem, Povah, Jensen and others, several of whom have isolated some 25-30 species from different soils, in different parts of the world; the genus seems to be especially abundant in cold climates.<sup>46</sup>

(b) Homothallic zygospores produced early and abundantly. Zygophores arise close together, always originating from a single aerial hypha; they are usually unequal. *Zygorhynchus* (No. 90, Pl. X). This genus is represented in the soil only by three species,<sup>47</sup> but these are among the

<sup>45</sup> Naumov, N. A. Material. Mikol. Fitopathol. (Russian), 6: 180-192. 1927.

<sup>46</sup> Nielson, N. Meddel. Gronland. Kopenhavn. 1927.

<sup>47</sup> Namyslowski, B. Ann. Mycol., 8: 152-155. 1910; Green, E. Ann. Bot. 41: 419-435. 1927.

very few most common soil fungi. They have been isolated by Hagem, Namyslowski, Jensen, and have been found in every soil examined from all parts of the world. They are found especially in sandy subsoils poor in organic matter.

- 2'. Sporangia similar to those of the Mucoraceae, but of two kinds: one kind is multispored, the membrane breaking up, leaving only a naked columella; the other kind of sporangia (sporangioles) contains few spores, which have persistent membranes and are often without columellae. THAMNIDIACEAE. Species of *Thamnidium* (*T. elegans*) have been isolated from the soil by Jensen, Dale, Pratt and others.
- 3'. Sporangia of one kind only, multispored; the membrane is for the major part solid, persistent, of a very dark blackish color or is swelling only toward the base. PILOBOLACEAE. *Pilaira anomala* has been isolated from soil by Oudemans and Koning; species of *Pilobolus* are commonly found on horse manure.
- 4'. Sporangia without a columella, with a diffuent disappearing membrane, as in the case of the Mucoraceae. Zygosporangia enclosed singly in a carposporium. MORTIERELLACEAE. *Mortierella* occurs abundantly on decomposing organic matter, some species being parasitic on other fungi. Several species have been found in the soil by Oudemans and Koning in Holland and by Nielson in Northern Greenland.
2. Reproduction asexually by conidia produced either solitary or in chains, sporangia not produced, suborder CONIDIOPHORAE.
  - 1'. Conidia solitary, spherical or oval, borne on conidiophores swollen in the middle or at the extremity. CHAETOCLADIACEAE.
    - (a) Conidiophores branched dichotomously in bunches or arranged irregularly. Round or oval conidia are borne around a spherical head. *Cunninghamella* (No. 93, Pl. X). *C. elegans* has been isolated from the soil by Lendner, Povah, and others; Paine<sup>48</sup> isolated from American soils another species, *C. verticillata*.
    - (b) Conidiophores verticillately branched, swollen into small heads furnished with sterile threads. *Chaetocladium*.
  - 2'. Conidia in chains:
    - (a) Conidiophores not swollen at tip. *Piptocephalis*.
    - (b) Conidiophores swollen at apex:
      - (a') Conidiophores not branched. *Syncephalis*.
      - (b') Conidiophores branched. *Syncephalastrum*.

The last 3 genera are only rarely found in the soil. Many of them are parasitic on the Mucorales.

B. ASCOMYCETES. Mycelium multicellular. The group is characterized by the formation of an ascus or sac which usually contains eight spores;

<sup>48</sup> Paine, P. S. *Mycologia* 19: 248-267. 1927.

these asci are assumed to represent a "perfect" stage, in some cases certainly developed subsequent to fertilization; the fruiting masses containing the asci are very variable.

I. PROTOASCI, without ascogenic hyphae. This group includes the yeasts, which reproduce vegetatively by budding.<sup>49</sup>

1. Cells not forming at once a surface membrane on liquid sugar media:

(a) Ascospores having a single membrane, cells not fusing in pairs before formation, spore germination by ordinary budding. *Saccharomyces*.

(b) Ascospores having two membranes. *Saccharomycopsis*.

2. Cells forming a surface membrane at once on sugar media: Ascospores lemon shaped or with pointed ends. *Willia*.

The presence of yeasts in the soils of orchards and vineyards has been established by Hansen<sup>50</sup> and others. Wine yeasts have been found in such soils even at a depth of 20 to 30 cm. below the surface, but not at 40 cm. depth. Soils rich in humus, such as peat soils, offer a favorable habitat for these organisms.<sup>51</sup> The greatest number of yeasts isolated from the soil<sup>52</sup> are wild yeasts, including also white and red species of *Torula*. An examination of eighty-seven soils from different parts of the United States revealed the presence of yeasts only in 45 per cent of the soils; only two soil samples gave more than one species.<sup>53</sup> In Germany, however, 52.5 per cent of the soils examined, especially field and orchard soils, contained yeasts.<sup>54</sup>

II. EUASCI, with ascogenic hyphae. The genus *Chaetomium* (No. 94, Pl. XI), among the *Sphaeriales*, is represented in the soil by various species isolated by Jensen, Traaen, Waksman, Abbot, Takahashi, Paine and others. It grows readily on manure, especially on mushroom composts; it seems to excrete a toxic substance which is highly injurious to the development of the cultivated mushroom, *Agaricus campestris*.

Among the *Sordariaceae*, the genera *Sordaria* and *Sporormia* have been isolated from the soil by Jensen. *Neonectria ramulariae*, among the *Hypocreales*, has been found in Texas soil by Werkenthin.

<sup>49</sup> A detailed study of the classification of yeasts and yeast-like fungi is given by Guilliermond—Tanner. The Yeasts; by Anderson, H. W. Jour. Inf. Dis., 21: 341-381. 1917; Kohl, F. G. Die Hefepilze. 1908.

<sup>50</sup> Hansen, E. C. Ann. Bot., 9: 549-560. 1895; Centrbl. Bakt. II, 10: 1-8. 1903; 14: 545-550. 1905; Compt. Rend. Trav. Lab. Carlsberg., 9: 61-69. 1911.

<sup>51</sup> Müller-Thurgau, H. Centrbl. Bakt. II, 14: 296-297. 1905; Lemmermann, Fischer, et al., 1909 (p. 244).

<sup>52</sup> Klöcker, A. Compt. Rend. Lab. Carlsberg., 7: 273-278. 1909; deKruyff, E. Centrbl. Bakt., II, 21: 616-619. 1908.

<sup>53</sup> Starkey, R. L. and Henrici, A. T. Soil Sci., 23: 33-46. 1927; Ciferri, R. Prof. First. Intern. Congr. Soil Sci. (1927), 3: 350-359. 1928.

<sup>54</sup> Nissen, W. Milchwirtsch. Forsch. 10: 30-67. 1930.

*Pyronema confluens*, among the *Pezizales*, grows very well on soil heated at 100°C. or burnt.<sup>55</sup>

Those *Aspergillaceae* which produce sexual stages or ascus fructifications belong to the *Plectascales*.

<sup>55</sup> Robinson, W. *Ann. Bot.* 40: 245-272. 1926.

## PLATE XI

### SOIL FUNGI—ASCOMYCETES AND HYPHOMYCETES

94. *Chaetomium olivaceum*: *A*, perithecium  $\times 40$ ; *B*, mature and immature asci,  $\times 160$ ; *C*, ascospores,  $\times 360$  (from Jensen).

95. *Trichoderma lignorum* (from Jensen).

96. *Trichoderma koningi*: *a*, hyphae with conidiophores,  $\times 56$ ; *b-c*, conidiophores with sterigmata; *e*, conidia  $\times 250$  (from Goddard).

97. *Sporotrichum poae* (from Schwarze).

98. *Acrostalagus cinnabarinus*: *a-b*, hyphae bearing conidiophores  $\times 56$ ; *c*, conidiophore with sterigmata; *d*, conidia,  $\times 250$  (from Goddard).

99. *Monilia koningi*: *b*, conidial fructification,  $\times 56$ ; *d*, same,  $\times 100$ ; *f*, sterigmata,  $\times 250$  (from Goddard).

100. *Cladosporium herbarum*: *a*, portion of colony grown in a hanging drop, showing mode of branching; *b*, part of the same to show arrangement of spores,  $\times 270$  (from Dale).

101. *Fusarium oxysporum*: showing conidia (*A, B, C, D*), chamydospores (*E, F, I*), and conidiophores (*G, H, J*) (from Sherbakoff).

102. Typical green *Penicillium*, *Pen. chrysogenum*: *c, d*, branching of conidial fructification,  $\times 600$ ; *k, j, m*, sketches of conidial fructifications,  $\times 90$  (from Thom).

103. *Penicillium purpurogenum*: *b*, typically verticillate branching at the apex of the conidiophore; *e*, conidia bearing cell or sterigma; *g*, diagrammatic representation of the entire conidial apparatus (from Thom).

104. *Penicillium*, soil series: colonies pale green, velvety at border, but more or less floccose in center with under side of mycelium rose to dark-red, conidia becoming glabrous, 2 to 3  $\mu$  in diameter (from Thom).

105. *Asp. terreus*: *a*, semidiagrammatic sketch of vesicle and sterigmata: *b, c, d*, primary and secondary sterigmata,  $\times 1000$ ; *e*, conidia,  $\times 1000$ ; *f*, diagram of stalk and base of calypttrate conidial mass (from Thom and Church).

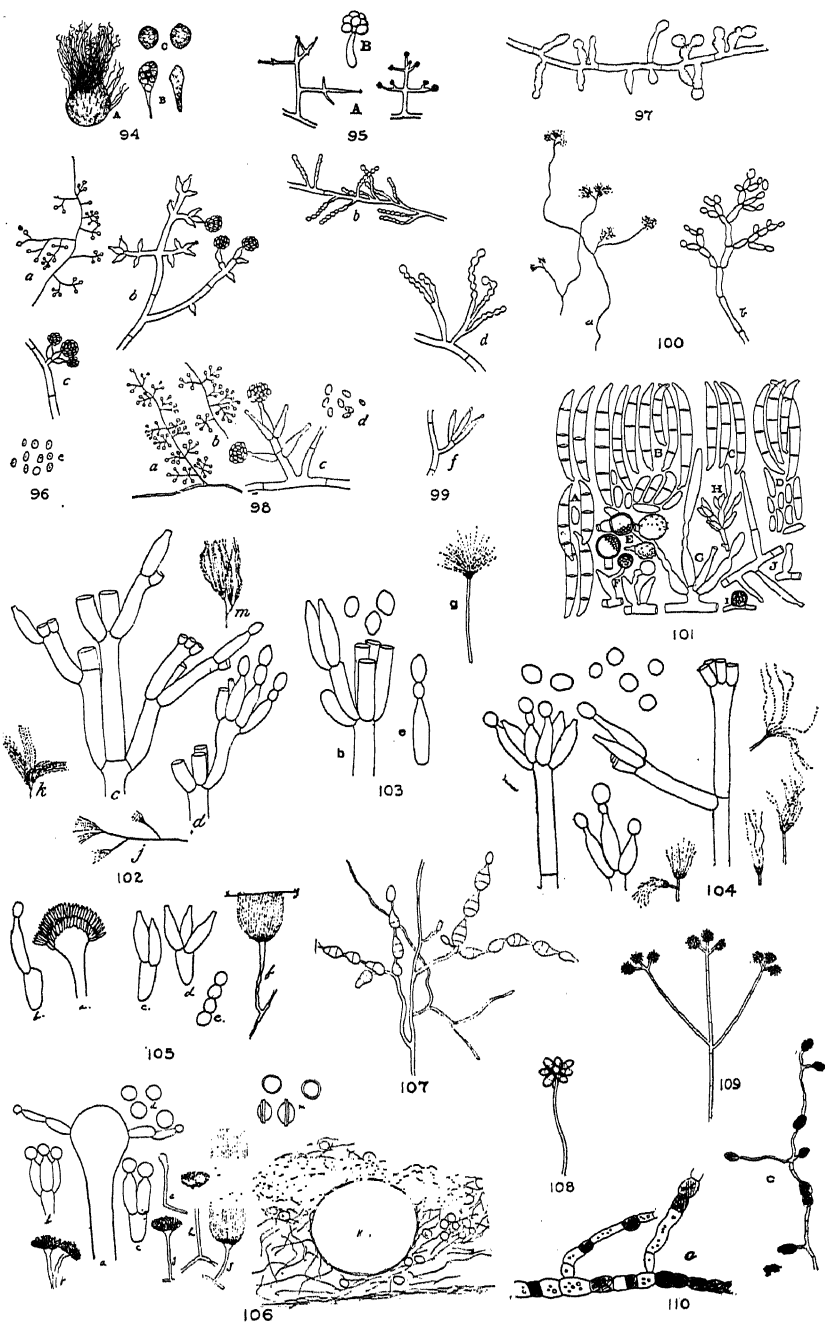
106. *Asp. nidulans*: *a*, diagrammatic section of vesicle with two sterigmata; *b, c*, primary and secondary sterigmata,  $\times 1000$ ; *d*, conidia  $\times 1000$ ; *e, f, g, h, j*, diagrams of stalks and heads; *k*, perithecium surrounded by sterile hyphae; *m*, group of ascospores (from Thom and Church).

107. *Alternaria humicola*, showing a portion of branching chains of spores,  $\times 150$  (from Dale).

108. *Cephalothecium roseum*, showing conidiophore and conidia (from Schwarze).

109. *Botrytis vulgaris* (from Schwarze).

110. *Dematiium*: *a*, showing intercalary dark cells; *b*, *Torula*-like type showing terminal dark cells on lateral branches,  $\times 270$  (from Dale).





## C. FUNGI IMPERFECTI, no sexual spore formation known.

I. HYPHOMYCETES.<sup>56</sup> Hyphae septate, hyaline or dark colored, separated from one another or united into coremia. Conidia are formed either as oidia, by the breaking up of hyphae, or on little differentiated branches of the mycelium, or on special conidiophores. The latter are simple or much branched. The order is divided into 4 families, according to shape of conidiophore and structure of mycelium.

1. Conidia produced on single conidiophores, more seldom in the form of oidia. Vegetative hyphae as well as conidia and conidiophores hyaline, pale or light colored, not dark. MUCEDINACEAE:

1'. Spores one-celled:

1''. Conidiophores never sharply differentiated from mycelium, sometimes lacking; conidia may develop by the breaking up of hyphae into oidia:

1'''. Conidia oval or spherical, never spindle-shaped:

(a) Conidiophores very short, hardly distinguished from the mycelium:

(a') Conidia produced on short side branches singly or one after another, *Myceliophthora*. Isolated from the soil by Goddard.

(b') Conidia large, with a thick membrane, *Coccospora*. Isolated from the soil by Goddard.

(b) Conidia developing as oidia by the breaking up of hyphae or as chains on short, not sharply differentiated branches, *Oidium* (*Oospora*). Isolated from the soil by Adametz, Dale, Jensen, and others.

(c) Conidia on definite branches; mycelium usually well developed and compact, *Monilia* (No. 99, Pl. XI). Various species have been isolated from the soil by Oudemans and Koning, Waksman and Abbott.

2'''. Conidiophores well defined, erect, short; conidia in chains, short cylindrical, truncate at both ends, *Geotrichum*. Seldom found in the soil.

2'. Conidiophores sharply differentiated from the mycelium:

1''' Conidiophores unbranched, or slightly branched, forming a head of branches and conidia:

1'''' Conidia single, not in chains:

(a) Conidiophores unbranched, with swollen tip:

<sup>56</sup> In the study of this group of fungi the system used by Lindau (Fungi imperfecti: Hyphomycetes. Rabenhorst's Krypt. Flora, Abt. 8 and 9. 1907-1910) has been followed. This work as well as that of Engler, A., and Prantl, K. (Die natürlichen Pflanzenfamilien. Leipzig. 1897-1907) will be found to be of great assistance in the identification of most representatives of the Hyphomycetes, except in the case of the genera *Aspergillus*, *Penicillium* and *Fusarium*, where special monographs are available.



- (a') Surface of terminal swelling definitely divided into hexagonal areas, *Rhaphalomyces*. Found in the soil by Beckwith.
- (b') Surface of terminal swelling not so divided, *Oedocephalum*. Rarely found in the soil.
- (b) Conidiophores simple, but not with swollen tip, or branched:
- (a') Conidiophores unbranched, seldom divided, conidia adjoined at tip, one after another, but all remaining united into a head:
- (a'') Conidia embedded in slime, *Hyalopus*. Rarely found in soil.
- (b'') Conidia not embedded in slime, *Cephalosporium*. Frequently found in the soil by Koning, Dale, Waksman and others.
- (b') Conidiophores branched:
- (a'') Conidiophores tapering to a point bearing a head, *Trichoderma* (Nos. 95-96, Pl. XI). One of the most common groups of soil fungi. Very active in the decomposition of cellulose. Species of this organism have been isolated from the soil in different parts of the world.
- (b'') Conidiophores with three or more fine spines, each of which bears a head, *Botryosporium*. Rarely found in the soil.
- 2'''' Conidia born in chains:
- (a) Conidiophores swollen at apex, *Aspergillus* (Nos. 105-106, Pl. XI). Common in the soil, represented by a number of species.<sup>57</sup>
- (b) Conidiophores not swollen at apex:
- (a') Conidiophores branched, branches more or less unequal and not radiating:
- (a'') Conidia not embedded in slime, *Penicillium* (Nos. 102-104, Pl. XI). One of the most common genera in the soil; represented by over 40 species, some of which are specifically soil forms.<sup>58</sup>
- (b'') Conidia embedded in slime, *Gliocladium*. Several species were isolated from the soil by Dale, Takahashi and Abbott.
- (b') Branches of conidiophore terminal, approximately equal and radiating, *Amblyosporium*. Rarely found in the soil.
- 2''' Conidiophores unbranched or branched, but branches and conidia not forming a terminal head:
- 1'''' Conidia born on simple or branched, but not whorled hyphae:
- (a) Conidia produced irregularly on the mycelium, or on short lateral branches, *Sporotrichum* (No. 97, Pl. XI). Commonly found in the soil, but insufficiently studied.

<sup>57</sup> Gilman and Abbott, 1927 (p. 221).

<sup>58</sup> See Dale, Jensen, Waksman, Gilman and Abbott; Zaleski, K. Bull. Acad. Polon. Cl. Sci. Math. Nat. B. (1927). 1928.

- (b) Conidia produced on definitely differentiated erect conidiophores, which are usually much branched:
  - (a') Conidia single, terminal, *Monosporium*. Isolated from the soil by Koning, Dale and others.
  - (b') Conidia are usually loosely grouped at tip, *Botrytis* (No. 109, Pl. XI). Represented in the soil by a number of species, some of which (*B. cinerea*) are cosmopolitan.
  - 2'''. Conidiophores branched in whorls:
  - (a) Conidia-bearing branches thick and flask-shaped; conidiophores with long sterile tips, *Pachybasium*. Isolated from the soil by Goddard.
  - (b) Conidiophores without sterile tip, conidia not produced on flask-shaped branches:
  - (a') Conidia not forming chains:
  - (a'') Conidia not embedded in slime, *Verticillium*. Represented in the soil by various species, some of which possess a strong cellulose decomposing power. Species of *Geomyces*, related to *Verticillium*, have been isolated from the soil by Traaen.
  - (b'') Conidia embedded in slime, *Acrostalagmus* (No. 98, Pl. XI). Frequently found in the soil.
  - (b') Conidia in terminal chains, *Spicaria*. Various species of this genus were isolated from the soil by Koning, Jensen and Abbott.
  - 3''. Conidia born on differentiated intercalary cells of the conidiophore:
  - (a) Cells, bearing conidia, with raised points for attachment of conidia, *Gonatobotrys*. Rarely found in the soil.
  - (b) Cells, bearing conidia, smooth, *Nematogonium*. Isolated from the soil by Koning and Dale.
  - 2'. Spores two-celled, conidia solitary:
  - (a) Conidia with both cells smooth:
  - (a') Conidia born on sides of conidiophores usually on inflated cells, not terminal, *Arthobotrys*. Rarely found in the soil.
  - (b') Conidia produced at tips of conidiophores, not lateral, conidia solitary or in heads, pear shaped, *Trichothecium*. Isolated frequently from the soil.
  - (b) Terminal cell of conidium enlarged and roughened, *Mycogone*. Isolated frequently from the soil.
  - 2. Vegetative hyphae either short, almost unnoticeable, often breaking up into spores, or as abundant moldy growth, septated, usually dark, seldom light colored (with dark spores). Conidiophores short, upright, simple or branched, dark colored. Conidia unseptated or variously septated, always dark colored, light only in exceptional cases (the mycelium and conidiophores are then dark).
- DEMATIACEAE.
- 1'. Conidia unicellular:
  - 1''. Mycelium little developed and breaking up into oidia, or

conidia formed on short lateral hyphae that are not well differentiated from the remainder of the mycelium; conidia in chains easily broken up, *Torula*. Various species of *Torula* were found in the soil by Koning, Dale, Pratt and others.

2''. Mycelium definitely developed, with well differentiated conidiophores:

(a) Conidia not in chains:

(a') Conidia in terminal heads:

(a'') Conidia developing directly from conidiophore or with very short sterigmata, *Synsporium*. Isolated from the soil by Dale.

(b'') Conidia on thick, long sterigmata, *Stachybotrys*. Several species were isolated from the soil.

(b') Conidia not in terminal heads; conidia prickly, *Zygodesmus*. Isolated from the soil only rarely.

(c') Single conidia produced on branches, irregularly produced on the sides of the mycelium, *Acremoniella*. Rarely found in the soil.

(b) Conidia in chains:

(a') Conidiophores unbranched, lateral, with terminal chain of spores, *Dematium* (No. 110, Pl. XI). Frequently found in the soil (Abbott).

(b') Conidiophores with branched chains of conidia, *Hormodendrum* (*Cladosporium*). Common in the soil, various species having been isolated by a number of investigators (No. 100, Pl. XI).

2'. Conidia two-celled:

(a) Conidiophores short, not well differentiated from the mycelium:

(a') Conidia solitary, *Dicoccum*. Rarely found in the soil.

(b') Conidia in chains, *Bispora*. Rarely found in the soil.

3'. Conidia more than two-celled:

(a) Septa of conidia perpendicular to long axis of spore, all parallel; conidia not in chains, *Helminthosporium*. Frequently found in the soil by Dale, Takahashi and Abbott.

(b) Septa of conidia both longitudinal and crosswise; conidiophores well differentiated:

(a') Conidia solitary and apical:

(a'') Conidiophores decumbent, formed in lateral branches of mycelium, *Stemphylium*. Various species have been isolated from the soil by Dale, Takahashi and others.

(b'') Conidiophores straight, more erect, conidium terminal, *Macrosporium*. Found in the soil by Dale and Pratt.

(b') Conidia in chains, *Alternaria* (No. 107, Pl. XI). Represented in the soil by several species.

3. Vegetative hyphae septate, branched, hyaline or dark colored. Conidiophores uniting in parallel strands to form upright coremia.

From the separation of hyphae, at the top of the coremium, the conidiophores either form a head or are slightly radiating.  
STILBACEAE:

- 1'. Hyphae, coremium, and conidia hyaline or light colored; conidia one-celled; coremium with a more or less definite head, conidia not born along entire side:

(a) Conidiophores scarcely diverging at top, *Cilicopodium*. Rarely found in the soil.

(b) Conidiophores divergent at top; each coremium with lateral heads as well as terminal, *Tilachlidium*. Found in the soil by Koning.

- 2'. Hyphae, coremium and conidia usually all dark:

(a) Conidia not in chains, ovoid to oblong, hyaline, *Graphium*. Rarely found in the soil.

(b) Conidia in chains, *Stysanus*. Found in the soil by Koning, Goddard and others.

4. Mycelium consists of branched, septated hyphae, growing in or on the medium; characteristic fructification; growth mostly of a waxy or slimy composition, often quite tough. TUBERCULARIACEAE.

(a) Conidia and hyphae hyaline and light colored; sickle shaped conidia, both ends more or less pointed, *Fusarium* (No. 101, Pl. XI).

One of the most common groups of soil fungi.<sup>59</sup> Various species of *Fusarium* were isolated from soils of different parts of the world. Many are parasitic on various plants but are also capable of leading a saprophytic existence in the soil. They are capable of decomposing cellulose and other organic complexes very actively.

(b) Conidia or hyphae dark or gray; conidiophores very short, conidia netted or prickly, *Epicoccum*. Rarely found in the soil.

A number of other genera of fungi belonging to the Hyphomycetes have been isolated from the soil. It is sufficient to mention *Ramularia*, *Pachybasium*, *Gliobotrys*, *Priconia*, *Mesobotrys*, *Spondyloccladium*, *Acrothecium*, *Velutella*, *Colletotrichum*, *Monascus*, etc.<sup>60</sup>

II. MELANCONIALES, *Melanconium*<sup>61</sup> is rarely found in the soil.

### III. SPHAEROPSIDALES:

*Chaetomella*. Found in the soil by Koning, Pratt and Abbott.

Species of *Phoma*, *Conisthyrium* were isolated from the soil by Waksman, Gilman and Abbott.

<sup>59</sup> Sherbakoff, 1915 (p. 222); Pratt, O. A. Jour. Agr. Res., 13: 73-99. 1918; Taylor, 1917 (p. 241); Appel, O. and Wollenweber, H. W. Arb. K. biol. Anst. Land. Forstw. 8: 1-207. 1913; Brown, W. Ann. Bot. 42: 285-304. 1928.

<sup>60</sup> Gilman and Abbott, 1927 (p. 221).

<sup>61</sup> Edgerton, C. W. Jour. Amer. Microscop. Soc., 31: No. 4. 1912.

## IV. Sterile mycelium:

## 1. Sclerotia formed:

(a) Sclerotia abundant, mycelium occupies secondary place, *Sclerotium*. Frequently found in the soil.

(b) Sclerotia seldom formed, *Rhizoctonia*. Various *Rhizoctonia*, especially *Rh. solani*, are frequently found in the soil.

2. No sclerotia formed; hyphae united in strands, *Ozonium* (Dale).

## D. BASIDIOMYCETES, characterized by the formation of a basidium, producing four sterigmata, each bearing a single spore.

I. Number of basidiospores indefinite. HEMIBASIDIOMYCETES: *Ustilaginales*, including the smuts.

## II. Number of basidiospores usually four:

1. Basidium septate. PROTOBASIDIOMYCETES: *Uredinales* or the rust fungi, *Auriculariales* including saprophytes and hemisaprophytes occurring on wood; *Tremellales*, occurring in the tropics.

2. Basidium continuous. AUTOBASIDIOMYCETES: The Autobasidiomycetes are by far the most common Basidiomycetes in soil. They are cosmopolitan in nature and form large fruiting bodies. They are subdivided into 2 groups:

1'. *Hymenomycetales*, with the hymenium (spore-bearing surface) exposed at maturity.

(a) Hymenium spread over spines. *Hydnaceae*.

(b) Hymenium spread over radiating plates or gills. *Agaricaceae*.

(c) Hymenium lining the interior of tubes, the mouths of which appear as pores. *Polyporaceae*.

(d) *Thelephoraceae*.

(e) *Clavariaceae*.

Among the Agaricaceae we find a number of saprophytic organisms, capable of making extensive development in soils and in composts including species of *Coprinus*, *Agaricus*, *Lenzites*, etc. The Polyporaceae contain the genera *Merulius*, *Poria*, *Polyporus*, *Boletus*, *Formes*, capable of active decomposition of wood, including the cellulose and some even attack the lignins.

2'. *Gasteromycetales*, with hymenium enclosed in a more or less globular body until after the spores are ripe. These include puffballs (*Lycoperdaceae*), earth balls (*Sclerodermaceae*) and other families.

A large number of Basidiomycetes develop in the soil in considerable abundance, some of them being completely subterranean in their development. This is true especially of soils rich in organic matter, such as forest soils. Some grow specifically in pastures, others in forests. The type of forest (deciduous or coniferous trees) influences markedly the nature of the fungus association. The reaction of the soil, abundance and nature of organic matter are also of great importance in modifying the mushroom. A number of Basidiomycetes form the so-called fairy rings<sup>62</sup> of deep-green grass found in pastures. Many are found

<sup>62</sup> Lawes, J. B., Gilbert, J. H. and Warrington, R. Jour. Chem. Soc. London. 43: 208. 1883; Bayliss, J. S. Jour. Econ. Biol. 6: 111. 1911; Shanz, H. L.

growing on manure. Some are capable of producing mycorrhiza on the roots of trees and other plants (p. 263).

*Occurrence of specific fungi in the soil.* Of the various genera of fungi found in the soil, the most common, both in number of species and in frequency of occurrence, are *Zygorhynchus*, *Penicillium*, *Trichoderma*, *Fusarium*, *Mucor*, *Aspergillus* and *Rhizopus*. This is clearly demonstrated in table 20, where an asterisk designates that a particular genus is represented by one or more species in one or more soils. Brierley<sup>63</sup> tabulated systematically all the fungi which have been recorded and described in soil investigations and found 56 species of *Phycomycetes* belonging to 11 genera; 12 species of *Ascomycetes* belonging to 8 genera; 197 species of *Fungi imperfecti* including *Actinomyces*, but not sterile mycelium, belonging to 62 genera.

Fungi are not limited in the soil to any particular depth, but occur at all depths, at least to a depth of four feet or more even in soils in humid regions. The numbers usually drop below the surface (upper six inches), but in the subsoil there does not seem to be a rapid diminution with greater depth. Some genera, like *Zygorhynchus*, occur abundantly in the sub-surface soils. The genus *Mucor* seems to be limited, however, largely to the upper soil layers.<sup>64</sup> The distribution of fungi in the soil is believed to depend upon the amount of moisture and the character of the soil.<sup>65</sup> The distribution of certain organisms in the soil, like *Fusarium*, has been ascribed to earthworms.<sup>66</sup> Various fungi are found in the soil at a depth of 1 to 44 inches; practically the same species were isolated from the intestinal canals of grubs and worms picked out from the soil and properly washed; it was, therefore, concluded that the grubs and earthworms are the carriers of spores of soil fungi. It is interesting to note that Takahashi isolated the species *Zygorhynchus mölleri* and *Trichoderma koningi* in Japan at lower depths, while just below the surface he found species of *Aspergillus*, *Penicillium*, *Mucor*, *Stemphylium*, and

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and Piemeisel, R. L. Jour. Agr. Res. 11: 191. 1917; Buller, A. H. R. Researches on fungi. v. 2. Longmans. London. 1922; Ramsbottom, J. Queckett Microsc. Club. (2) 15: 231-242. 1927.

<sup>63</sup> Brierley, W. B. The microorganisms of the soil, by Sir John Russell. 1923, 118-130; see also Abbott<sup>60</sup> and Jensen, H. L. Soil Sci. 31: 123-158. 1931.

<sup>64</sup> Samutsevitch, M. M. Mater. Mikol. Fitopath. (Russian), 6: 204-213. 1927.

<sup>65</sup> Beckwith, T. D. Phytopathol., 1: 169-176. 1911.

<sup>66</sup> Taylor, M. W. Phytopathol., 7: 374-378. 1917; Rathbun, A. E. Phytopath. 8: 469-483. 1918.

TABLE 20  
*List of the more common genera of soil fungi found in different parts of the world*

GENUS	OUDEMANS AND KONING		DALE	JENSEN	GODDARD <sup>67</sup>	MCLEAN AND WILSON <sup>68</sup>	WAKSMAN	ABBOTT <sup>69</sup>	PRATT	TAKAHASHI <sup>70</sup>
	Holland	England	New York	Michigan	New Jersey	New Jersey	Iowa	Idaho	Japan	
<i>Absidia</i> .....	*	*	—	—	—	—	*	*	*	—
<i>Acrostalagmus</i> .....	*	—	*	*	*	—	*	*	—	*
<i>Alternaria</i> .....	*	*	*	—	*	*	*	*	—	*
<i>Aspergillus</i> .....	*	*	*	*	—	—	*	*	*	*
<i>Botrytis</i> .....	*	*	*	—	—	—	*	—	—	—
<i>Cephalosporium</i> .....	*	—	—	—	—	—	*	—	—	—
<i>Chaetomium</i> .....	—	—	*	—	—	—	*	*	*	—
<i>Fusarium</i> .....	—	*	—	*	*	—	*	*	*	—
<i>Hormodendrum</i> .....	—	*	*	*	*	*	*	*	—	—
<i>Mucor</i> .....	*	*	*	*	*	*	*	*	*	*
<i>Penicillium</i> .....	*	*	*	*	*	*	*	*	*	*
<i>Rhizopus</i> .....	—	*	*	*	*	*	*	*	*	*
<i>Stemphylium</i> .....	*	—	—	—	—	—	—	—	*	—
<i>Thamnidium</i> .....	—	*	*	—	—	—	—	—	*	—
<i>Trichoderma</i> .....	*	*	*	*	*	*	*	*	*	*
<i>Trichothecium</i> .....	—	*	*	—	—	—	—	—	—	—
<i>Verticillium</i> .....	—	*	—	*	*	—	*	—	*	—
<i>Zygorhynchus</i> .....	—	*	*	—	—	*	*	*	*	*

<sup>67</sup> Goddard, H. M. Bot. Gaz., **56**: 249-305. 1913.

<sup>68</sup> McLean, H. C., and Wilson, G. W. N. J. Agr. Exp. Sta. Bul., **270**: 39. 1914.

<sup>69</sup> Abbott, E. V. Bull. 194, Ia. Agr. Exp. Sta. 1926; Iowa State Coll. Jour. Sci. **1**: 15-36, 235-345. 1927.

<sup>70</sup> Takahashi, R. Ann. Phytopathol. Soc. Japan, **12**: 17-22. 1919 (Bot. Abstr. **5**: 92. 1920).

*Chaetomium*. However, the soils at very high elevations contain practically the same fungi as those found in the plains.<sup>71</sup>

In addition to the more than 60 genera of lower or filamentous fungi reported to be found in the soil, probably twice as many more could be demonstrated; these occur, however, to a comparatively more limited extent. The presence of a certain organism in large numbers need not indicate its great abundance in the soil, but may be due to abundant spore formation or to local development. Repeated isolation of an organism from different soils and from various parts of the same soil is essential before any claim can be laid to its active part in soil transformations.

As to the occurrence of Basidiomycetes in soil, most of the studies were based upon surface observations and not upon isolations from soil and cultivation in laboratory. Hence only those fungi were reported that produce fruiting stages visible to the naked eye. Gilbert<sup>72</sup> found that the nature and concentration of organic matter in the soil influences more than any other factor the development of these fungi. The reaction of the soil, moisture content, light, temperature, season of year, topography, nature of higher plant population, are among the other factors of importance in this connection. Some of the organisms are highly specialized, growing only under specific conditions and upon very few organic materials, while others are less specific growing under a great variety of conditions.

The higher fungi were divided<sup>72</sup> into two general groups: 1. *Calcofilic fungi*, including *Amanita ovoidea*, *A. verna*, *Lepiota granulosa*, *Clitocybe geotropa*, *Tricholoma album*, *Russula maculata*, *Cortinarius fulgens*, *Boletus satanas*, *Clavaria flava*, *Lycoperdon caelatum*, and numerous others; 2. *Calcofugic fungi*, including *Amanita virosa*, *Lepiota procera*, *Clitocybe clavipes*, *Lactarius turpis*, *Russula amoena*, *Cortinarius mucosus*, *Boletus bovinus*, etc.

On the basis of their relation to organic matter, the fungi were divided into seven groups: 1. *humicolous* forms, which grow on practically pure humus; 2. *terrestrial (geophilic, terricolous)* forms, which grow in soil

<sup>71</sup> Scaramella, P. Boll. Sez. Ital. Soc. Intern. Microb. 2: 478-482. 1930. Further information on the occurrence of fungi in different soils is found in the work of Janke, A. and Holzer, H. Centrbl. Bakt. II, 79: 50-74. 1929; Swift, M. E. Mycolog. 21: 204-221, 1929; Raillo, A. Centrbl. Bakt. II, 78: 515-524. 1929; Thakur, A. K. and Norris, R. V. Jour. Ind. Inst. Sci., 11A, Pt. 12: 141-160. 1928; Jensen, H. L. Soil Sci. 31: 123-158. 1931.

<sup>72</sup> Gilbert, E. J. La mycologie sur le terrain. François, Paris. 1928.



containing more or less organic matter; 3. *coprophilic* (*fimicolous*) forms, growing on manure, this being a special type of group 1; 4. *hypogeous* forms, which grow below the surface of the soil, being a type of group 2; 5. *lignicoleous* forms, growing on the lignins of plant materials; 6. *pseudo-parasitic* forms (wound parasites, mycorrhiza formers, facultative parasites), and 7. *true parasites*.

Fungi are found in peat bogs and swamps only to a limited extent.<sup>73</sup> The nature of the coprophilous fungus flora, or organisms living primarily on manure, will be discussed elsewhere, as well as the flora of forest soils.

*Activities of fungi in the soil.* Fungi take part in at least two important soil processes: (1) rapid decomposition of complex organic substances; (2) assimilation of soluble inorganic nitrogen compounds and minerals, especially in the presence of available energy, thus removing them temporarily from the soil solution. The addition of fresh stable manure rich in straw, of green manures, and of other plant residues to the soil greatly stimulates the development of fungi; the nature of the organisms developing most abundantly depends to a large extent upon the constituents of the organic matter added. The addition of stable manure was found to stimulate the development of *Penicillia* and especially of *Mucorales* and *actinomyces*.<sup>74</sup> It came to be generally recognized<sup>75</sup> that fungi are associated in the soil largely with the organic matter. The addition of pure cellulose, especially in the presence of available nitrogen, brings about an extensive development of various fungi, such as *Trichoderma*, *Fusarium*, *Verticillium*, *Monosporium*, certain *Penicillia*, and other cellulose decomposing organisms. The reaction of the soil, the moisture content, and the nature and amount of the available nitrogen greatly modify the types of fungi developing in the soil as a result of the addition of celluloses or cellulose-rich materials. Plant substances are commonly added to the soil in the form of residues or green manures, which contain only a very small amount of nitrogen (0.3 to 2.0 per cent). Fungi rapidly decompose practically all the constituents of the organic matter added to the soil, with the possible exception of the lignins. They assimilate as much as 30 to 50 per cent of the carbon for the synthesis of cell substance. The latter contains 3.5 to 8.0 per cent of nitrogen. In other words the minimum nitrogen content

<sup>73</sup> Thaysen, Baker and Bunker. *Biochem. Jour.* **20**: 210-216. 1926.

<sup>74</sup> Lemmermann, O. and Fischer, H., Kappen, H. and Blanck, E. *Landw. Jahrb.*, **38**: 319-364. 1909.

<sup>75</sup> Elliott, J. S. B. *Ann. Appl. Biol.* **17**: 284-305. 1930.

of the fungus mycelium is twice as much as the maximum nitrogen content of green manure. If one part of fungus mycelium is synthesized for every three parts of green manure and plant stubble decomposed, and if the former contains three or more times as much nitrogen as the latter, this element will be completely reassimilated by the fungi; they may even assimilate, under certain conditions (when the plant material is low in nitrogen), the available nitrogen compounds in the soil.

The rôle of fungi in the growth of higher plants may thus be both beneficial and injurious, depending upon conditions. This can be well illustrated by the phenomenon of formation of "fairy rings." When the spores of *Agaricus* germinate in the soil, small circular areas are formed and the native grasses are stimulated. The mycelium begins to spread in all directions as fungi usually do in culture media. The outward

TABLE 21  
*Influence of reaction upon the growth of fungi*

ORGANISM	CRITICAL pH VALUES
<i>Mucor glomerula</i> .....	3.2-3.4 to 8.7- 9.2
<i>Asp. oryzae</i> .....	1.6-1.8 to 9.0- 9.3
<i>Asp. terricola</i> .....	1.6-1.8 to 9.0- 9.3
<i>Pen. italicum</i> .....	1.9-2.2 to 9.1- 9.3
<i>Pen. variable</i> .....	1.6-1.8 to 10.1-11.1
<i>Fus. bullatum</i> .....	2.0-2.2 to 9.2-11.2
<i>Fus. oxysporum</i> .....	1.8-2.0 to 9.2-11.1

growth is slow—about 12 cm. a year. The sod is at first stimulated by an increase in the available nitrogen resulting from the decomposition of the organic matter in the soil, then killed by insufficient soil moisture in the area of dense mycelium. When the mycelium in its turn begins to decompose, the native grasses again invade the soil and develop luxuriantly, because of the abundant supply of readily available nitrogenous materials.<sup>76</sup>

*Influence of reaction upon the growth of fungi.* A detailed discussion of the various phases of the physiology of the fungi is out of place here, since it is given in the standard texts on plant physiology. Attention may be called to only some physiological properties of fungi which are important from the point of view of the growth and activities of these organisms in the soil.

<sup>76</sup> Shanz and Piemeisel, 1917 (p. 240).

It has been pointed out above that acid soil conditions favor the development of fungi. It need not, however, be construed that fungi grow only under acid conditions or even that they have their optimum growth at distinctly acid reactions; they have a rather wide range of reaction optimum, as shown in table 21.<sup>77</sup> In the case of Basidiomycetes, the range of pH in which they will grow and amount of mycelium produced will depend upon the individual organism, composition of the medium, initial acidity and temperature. In synthetic solutions, they will grow at pH 2.5-3.4 to 7.0-7.6; in organic media, the same organisms will grow at pH 2.0-3.0 to 7.5-8.5.<sup>78</sup>

Fungi are thus shown to be much more resistant to acidity than the other groups of soil microorganisms. On the alkaline side, however, they are not more resistant than the bacteria. On the acid side there will, therefore, be no competition for the available plant food.

The reaction of the medium has an important influence upon the germination of fungus spores and upon the respiration of the organisms.<sup>79</sup> Increasing acidity favorably influences the germination of the spores; a maximum of germination is exhibited by the majority of the spores tested at a pH of 3.0 to 4.0. Inhibition of germination is evidenced only at pH 1.5 to 2.5; the alkaline limits vary with the organism and with the medium.

Fungi as a rule modify greatly the reaction of the medium, by the production of organic acids from available carbohydrates, by the consumption of organic acids (leaving the medium less acid), or by the formation of ammonia from proteins.<sup>80</sup>

*Cellulose decomposition by fungi.* Koning<sup>81</sup> was the first to point out the great abundance of fungi in forest soils, where colorless and brown, septated and non-septated mycelium is found to penetrate the whole mass of organic matter. Koning suggested that fungi play an important rôle in the soil in decomposing the organic matter and in transforming it into humus. Under favorable conditions and in the presence of available energy, fungi grow very rapidly in the soil and produce a greater amount of CO<sub>2</sub> than do bacteria.<sup>82</sup> This led Neller to conclude that

<sup>77</sup> Johnson, H. W. Iowa Agr. Exp. Sta. Res. Bul. 76. 1923.

<sup>78</sup> Wolpert, F. S. Ann. Mo. Bot. Gard. 11: 43-97. 1924.

<sup>79</sup> Webb, R. W. Ann. Mo. Bot. Gard., 8: 283-341. 1921; Molliard, M. Compt. Rend. Soc. Biol., 83: 50-51. 1920.

<sup>80</sup> Butkewitsch, Wl. Jahrb. wiss. Bot., 38: 147-240. 1903.

<sup>81</sup> Koning, C. J. Arch. neerland. Sci. Exact. Nat., Ser. II, 9: 34-107. 1904.

<sup>82</sup> Neller, J. R. Soil Sci., 5: 225-241. 1918; Potter, R. S. and Snyder, R. S. Soil Sci., 5: 359-377. 1918.

fungi exist in the soil not merely in the form of spores, but are active there, since the  $\text{CO}_2$  production by pure cultures of fungi approached more that of a normal soil than the  $\text{CO}_2$  produced under similar conditions by bacteria. The decomposition of cellulose and of allied compounds in the soil by fungi is of great importance in soil fertility. This accounts for their abundance in soils rich in organic matter and for the great increase in numbers when stable manure and green manure are added to the soil. The addition of 1 per cent of cellulose to the soil in the form of pure filter paper may result in an increase in the number of fungi from 50,000 to 1,250,000 per gram in two weeks. It has been further found that when a soil to which cellulose is added is treated with a volatile antiseptic, in sufficient amounts to kill the fungi, cellulose decomposition is greatly reduced. This process was found to take place under aerobic conditions, parallel with the development of fungi; in other words, these organisms are important agents in the breaking down of the most abundant constituent of plant organic matter.

Students of plant diseases observed in the sixties of the nineteenth century that fungus hyphae grow in plant tissues, thereby penetrating cell walls. Hartig<sup>83</sup> found that, in trees affected by fungi, all tissues disappear partly or entirely. De Bary<sup>84</sup> was the first to claim that *Botrytis vulgaris* can decompose cellulose; actually he demonstrated that this organism can dissolve the middle lamella, which was not distinguished from cellulose by the older anatomists. Behrens<sup>85</sup> first used filter paper as a nutrient for fungi and established the fact that *Botrytis cinerea*, *Sclerotinia libertiana*, *Botrytis vulgaris*, and a pseudo-Demato-phora were able to derive their energy from pure cellulose. Only about 10 per cent of the paper was decomposed; this led Schellenberg<sup>86</sup> to suggest that only the impurities or hemicellulose-like compounds were decomposed.

Van Iterson<sup>87</sup> inoculated, with soil or humus, filter paper moistened with a solution of 0.05 per cent ammonium nitrate and 0.5 per cent of  $\text{KH}_2\text{PO}_4$  in tap water. He isolated 35 fungi including the genera *Sporotrichum*, *Chaetomium*, *Botrytis*, *Stachybotrys*, *Cladosporium*, *Tricho-*

<sup>83</sup> Hartig. Untersuchungen über die Zersetzungserscheinungen des Holzes. Berlin, 1878.

<sup>84</sup> de Bary, A. Bot. Ztg., 44: 377, 420. 1886.

<sup>85</sup> Behrens, J. Centrbl. Bakt. II, 3: 584, 639, 743. 1897; 4: 514, 547, 577, 635, 700, 739, 770. 1898.

<sup>86</sup> Schellenberg, H. C. Flora, 98: 257-308. 1908.

<sup>87</sup> van Iterson, C. Verslagen d. k. Akad. Wetensch., 9: 807-820. 1903; Centrbl. Bakt. II, 11: 689-698. 1904; Koning, 1904 (p. 246).

cladium, Mycogone, which are capable of decomposing cellulose. Not more than 4 to 14 per cent of the paper was decomposed. These results were subject to criticism, since tap water, which may contain various impurities, was used. The comparatively small loss in the weight of the cellulose was ascribed to the hemicelluloses present in the cellulose source used. *Fusarium vasinfectum* and other species of *Fusarium* were found<sup>88</sup> capable of transforming as much as 50 to 80 per cent of the cellulose, in the form of filter paper, into soluble forms; the medium used consisted of 10 gm. of paper and 50 cc. of a synthetic solution ( $\text{KNO}_3$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{MgSO}_4$ ) placed in Erlenmeyer flasks. As late as 1908, however, both Schellenberg and Froehlich<sup>89</sup> claimed that, with the possible exception of *Botrytis*, *Fusarium* and wood destroying fungi, it has not been demonstrated as yet that fungi are capable of decomposing cellulose. It remained for the more recent investigators<sup>90</sup> to establish definitely that not only do fungi decompose cellulose, but that pure cultures of fungi will decompose quantitatively within 3 to 4 weeks 50 per cent or more of the cellulose added in the form of filter paper. The addition of cellulose to the soil brings about an extensive development of fungi, most of which possess a very strong cellulose decomposing power. These include various species of *Penicillium*, *Aspergillus*, *Trichoderma*, *Sporotrichum*, *Fusarium*, and other forms which are able to decompose cellulose. McBeth<sup>91</sup> suggested that, in moist soils, particularly in humus soils, the fungi play a much more important part than in dry soils. Daszewska found *Verticillium cellulosa*, *V. glaucum*, *Sporotrichum olivaceum* and various other *Sporotricha*, *Fusaria*, *Monosporia*, *Alternaria* and *Monilia* among the strongest cellulose decomposing fungi in the soil. She also concluded that the *Hyphomycetes* play a much more important part than the bacteria in the decomposition of cellulose in the soil, the color of the humus being due to the color of the mycelium and the spores of fungi. Sugars and alcohols were formed as intermediary products.

<sup>88</sup> Appel, O. and Schikhorra. Arb. K. biol. Anst. Land. u. Forstw., 5: 155-188. 1906.

<sup>89</sup> Froehlich, H. Jahrb. Wiss. Bot., 45: 256-302. 1908.

<sup>90</sup> Koning, C. J. Verlesungen v. de gewone Vergad. d. Wis. e. nat. Afdelling. Nov. 1912; Daszewska, W. Bull. Soc. Bot. Genève, ser. 8, fas. 8, 255-316. 1913 McBeth, I. G. and Scales, F. M. U. S. Dept. Agr. Bur. Plant Indus. Bul. 266, 1913; Scales, F. M. Bot. Gaz., 60: 149-153. 1915; Otto, H. Inaug. Diss. Berlin. Borntraeger. 1916.

<sup>91</sup> McBeth, 1916 (p. 192).

Otto<sup>90</sup> investigated a series of soil fungi and found the following to be able to decompose true cellulose actively: Species of *Stemphylium*, *Mycogone*, *Stachybotrys*, *Trichoderma*, *Cladosporium* (*Hormodendrum*), and *Penicillium*. The cellulose was decomposed by the fungi by means of hydrolytic enzymes, which are produced only in the presence of cellulose in the medium. Further information on cellulose decomposition by fungi is given elsewhere.<sup>92</sup>

The Mucorales are as a rule unable to decompose cellulose and most hemicelluloses, but are able to decompose pectins, whereas *Botrytis* and other fungi decompose the fiber itself.<sup>93</sup> Many of the Basidiomycetes are capable of decomposing cellulose. Here belong those organisms which take an active part in the decomposition of wood in forests, as well as timber in general, causing the so-called "brown-rots." On the other hand, other Basidiomycetes can decompose both lignin and cellulose, causing the "white rots," as shown elsewhere (p. 401). Hymenomycetes produce various enzymes capable of hydrolyzing hemicelluloses to sugars.<sup>94</sup>

According to the earlier investigators fungi are the proper humus builders in the soil. The fallen leaves, at the end of the vegetative period in the fall, are found to be penetrated with fungus mycelium, which decomposes the leaves readily, with the production of humic substances. These accumulate, because they cannot serve as a source both of carbon and of nitrogen<sup>95</sup> but, in the presence of available sources of carbon, they can be used as sources of nitrogen by fungi. More recent information tends to show that, although fungi decompose most of plant residues (with the exception of lignins which are attacked only by certain specific organisms) completely, they synthesize extensive protoplasm, which is an important part of the soil organic matter.

*Decomposition of nitrogenous substances by fungi (ammonia formation).* Just as in the decomposition of cellulose and allied compounds, fungi play an important rôle in the decomposition of organic nitrogenous compounds. In the presence of available carbohydrates, the fungi utilize the nitrogen compounds only as sources of nitrogen; in the com-

<sup>92</sup> Heller, F. Inaug. Diss. Rostock. 1917; Hopfe, A. Centrbl. Bakt. I, 83: 374-386, 531-537. 1919; Traaen, 1914 (p. 221); Jensen, H. L. Soil Sci. 31: 123-158. 1931.

<sup>93</sup> Behrens, J. Centrbl. Bakt. II, 10: 524-530. 1903; Waksman and Skinner, 1926 (p. 185).

<sup>94</sup> Lutz, L. Compt. Rend. Acad. Sci. 190: 892-895. 1930.

<sup>95</sup> Reinitzer, F. Bot. Ztg., 58: 59-73. 1900; Nikitinsky, J. Jahrb. Wiss. Bot. 37: 365-420. 1902.

plete or relative absence of available carbohydrates, they utilize the nitrogenous substances as sources of carbon and of nitrogen. In view of the fact that the energy requirements of the fungi are greater than their nitrogen requirements, a great deal more of the protein molecule will be broken down to supply the necessary carbon. The excess of nitrogen present in the protein molecule over that required by the fungus for the building up of its own proteins will be left as a waste product, in the form of ammonia. In general, fungi play an important part in the mineralization of the organic matter, whereby the nitrogen compounds and minerals are liberated in inorganic forms; a part of these is used by the fungi for the synthesis of fungus proteins.

Müntz and Coudon and Marchal<sup>96</sup> pointed out, in 1893, the abundant formation of ammonia by fungi; the latter even ascribed the ammonia production in soils (particularly acid soils) chiefly to the action of fungi. Cyanamide is decomposed, with the formation of ammonia, as are urea, uric acid, and glycocoll.<sup>97</sup> According to McLean and Wilson, filamentous fungi are capable of producing a greater accumulation of ammonia from proteins than bacteria. All the organisms studied, including representatives of the families of Mucoraceae, Aspergillaceae, Moniliaceae and Dematiaceae, were found to be capable of producing ammonia from dried blood and from cottonseed meal. The Moniliaceae were most active. In 8 to 10 days, *Trichoderma koningi* liberated as ammonia over half of the nitrogen in dried blood (1 per cent in sterile soil). Species of *Aspergillus* and *Penicillium* formed the least amounts of ammonia from proteins. The addition of soluble phosphate stimulated in most cases the amount of ammonia accumulated. Most fungi were capable of allowing greater accumulations of ammonia from dried blood than from cottonseed meal. This is probably due to the fact that the latter is richer in available carbon compounds, which will allow a greater synthesis of fungus proteins with the decomposition of proportionally less protein of the cottonseed meal.

*Utilization of nitrogen compounds by fungi.* Soil fungi may assimilate the readily available nitrogen compounds of the soil, in the presence of favorable sources of energy, thus exerting a very unfavorable action upon the growth of higher plants. According to Rothe,<sup>98</sup> fungi exceed

<sup>96</sup> Müntz, A. and Coudon, H. Compt. Rend. Acad. Sci., **116**: 395-398. 1893; Marchal, E. Bul. Acad. Roy. Sci. Belg., **25**: 727-771. 1893.

<sup>97</sup> Kappen, H. Centrbl. Bakt. II, **26**: 633-643. 1910; Kossowicz, A. Ztschr. Gärungsphysiol., **1**: 60. 1912.

<sup>98</sup> Rothe. Inaug. Diss. Königsberg. 1904.

the bacteria and actinomyces, in acid as well as in neutral media, in the assimilation of available nitrogen and in storing it away as microbial organic matter; in the presence of  $\text{CaCO}_3$ , large quantities of nitrogen added to the soil in the form of ammonium salts are transformed by these organisms into very insoluble nitrogen compounds. The competition between fungi and higher plants for the available nitrogen, under certain conditions, was also pointed out by Hall and associates.<sup>99</sup> Hagem<sup>100</sup> found that Mucorales will readily assimilate ammonium salts and transform them into microbial proteins. As pointed out above, with cellulose or other carbohydrates as sources of energy, the fungi may reassimilate 30 to 40 per cent of the carbon of the substrate decomposed. This necessitates a parallel assimilation of nitrogen; about one unit of available nitrogen is transformed into microbial protein for every 30 units of cellulose decomposed. This leads to a considerable reduction of the available nitrogen in the soil.

Ehrenberg<sup>101</sup> stated that fungus protein is much less available for further decomposition than bacterial protein, the fungus spores containing a large quantity of nitrogen stored away in an unavailable form, to some extent in the form of chitin, not readily subject to decomposition. Other investigations seem to show, however, that a large part at least of the cell substance synthesized by fungi is as rapidly decomposed as organic substances of animal origin.<sup>102</sup> The disappearance of the available nitrogen added to the soil in the form of ammonium salts and nitrates is to be looked for more in the development of fungi than of bacteria. This is particularly true when these nitrogenous fertilizers are added together with large quantities of manure or straw, since the available energy introduced into the soil will allow a rapid growth of the fungi, with the result that available nitrogen compounds are used up by them, to the detriment of the growth of higher plants. This action of the soil fungi has also a favorable side, namely the temporary storing of the available nitrogen salts in an insoluble form, thus preventing their leaching by drainage and irrigation. The favorable and unfavorable effect depend upon the presence or absence of higher plants.

<sup>99</sup> Hall, A. D., Miller, N. H. and Gimmingham, C. T. *Proc. Roy. Soc. (London)*, B, **80**: 196-211. 1908.

<sup>100</sup> Hagem, 1908 (p. 221).

<sup>101</sup> Ehrenberg, P. *Mitt. Landw. Inst. Breslau*, **4**: 47-300. 1907; Wettstein, F. *Sitz. Ber. Akad. Wiss. Wien, Math. Nat. Kl. (I)*, **130**: 3-20. 1921 (*Centrbl. Bakt.* II, **58**: 329. 1923).

<sup>102</sup> Starkey, R. L. *Soil Sci.* **17**: 293-314. 1924.



The nutritive value of nitrogen compounds for fungi depends on the rapidity with which they can be transformed into amino acids.<sup>103</sup> Other investigators<sup>104</sup> are, however, of the opinion that the amino acids and nitrates are reduced to ammonium salts before they are assimilated by fungi. The ability of an organism to assimilate ammonium salts is in direct relation to its ability to withstand the mineral acid liberated.<sup>105</sup> Assimilation of nitrates by fungi usually takes place through the reduction of nitrates to nitrites and ammonia. Organisms, like certain Mucorales, that are incapable of reducing the nitrate molecule cannot assimilate this source of nitrogen.<sup>106</sup>

*Nitrogen-fixation.* Various claims have been put forth, at different times, that fungi are able to assimilate atmospheric nitrogen. In most cases the quantities fixed were very small, amounting to a few milligrams, so that doubt might arise whether this was not due merely to experimental errors. In some cases the mere fact that fungi grew on agar free from nitrogen compounds was taken as an index of positive nitrogen-fixation, the fact being overlooked thereby that some of these organisms can readily assimilate traces of ammonia present in the atmosphere and that various chemicals may contain, as impurities, small amounts of nitrogen. The more careful studies of recent investigators<sup>107</sup> have definitely established the fact that common soil fungi are unable to fix atmospheric nitrogen. The only possible exceptions to this rule

<sup>103</sup> Czapek, 1901-1902 (p. 365); Puriewitsch, K. *Biochem. Ztschr.*, **38**: 1-13. 1912.

<sup>104</sup> Raciborski, M. I. *Anz. Akad. Wiss. Krakau, Math. Naturw. Kl.*, p. 733. 1906; Hagem, 1910 (p. 221); Abderhalden, E. and Rona, P. *Ztschr. physiol. Chem.*, **46**: 179-186. 1910.

<sup>105</sup> Ritter, G. *Ber. deut. bot. Gesell.*, **25**: 255; **27**: 582-588; **29**: 570-577. 1908-1911.

<sup>106</sup> A detailed study of the nitrogen utilization by fungi has been made by Brenner, while the influence of environmental conditions on the activities of soil fungi has been reviewed by Coleman. Further information on the physiology of fungi including curves of growth, influence of temperature, reaction and concentration is given by Müller. The antagonistic action of fungi to one another was studied by Nadson and Zolkiewicz and Porter. Brenner, 1914 (p. 225); Coleman, D. A. *Soil Sci.*, **2**: 1-66. 1916; Müller, K. O. *Beitr. Allg. Bot.* **2**: 276-322. 1922; Nadson, G. A. and Zolkiewicz, A. I. *Bull. Jard. Bot. Rep. Russe.*, **21**: suppl. 1. 1921; Porter, C. L. *Amer. Jour. Bot.*, **11**: 168-188. 1924.

<sup>107</sup> Goddard, 1913 (p. 242); Chambers, C. O. *Plant World*, **19**: 175-194. 1916; Duggar, B. M., and Davis, A. R. *Ann. Mo. Bot. Gard.*, **3**: 413-437. 1916; see, however, Senn, G. *Biol. Rev. Biol. Proc. Cambridge Phil. Soc.* **3**: 77-91. 1928; Schober, R. *Jahrb. Wiss. Bot.* **72**: 1-105. 1930.

may be in the case of certain mycorrhiza fungi,<sup>108</sup> especially organisms belonging to the genus *Phoma* (*P. betae* and *P. radicis*), where positive nitrogen fixation has been demonstrated. Nitrogen fixation by yeasts was also found to be negative.<sup>109</sup>

The possibility that certain fungi, like *Pen. luteum*, are capable of oxidizing sulfur has also been suggested.<sup>110</sup>

<sup>108</sup> Peklo, J. Ztschr. Gärungsphysiol., **2**: 275-289. 1913; Ternetz, C. Jahrb. wiss. Bot., **44**: 353-408. 1907.

<sup>109</sup> Kossowicz, A. Biochem. Ztschr., **64**: 82. 1914.

<sup>110</sup> Abbott, E. V. Soil Sci., **16**: 207-216. 1923; Rippel, A. Centrbl. Bakt. II, **2**: 290-295. 1924.

## CHAPTER XI

### MYCORRHIZA FUNGI

*Nature of mycorrhiza formation.* In addition to the true saprophytic fungi, which live only upon the tissues of dead plants and animals, the soil harbors obligate and facultative parasitic fungi, which are capable of attacking living plants, reducing their vitality, destroying their tissues and frequently causing the death of the plant, and *mycorrhiza fungi*. The latter are capable of attacking the subterranean organs of the plants, feeding upon the plant constituents; the plant cells may recover, however, and in their turn digest the fungus mycelium; in this instance, the subterranean part of the plant and the fungus form an association which is frequently of benefit to both, this union being known as *mycorrhiza* or *fungus-root*. This term was applied fifty years ago and is still used, although it is known that the hyphae of the fungus penetrate, in addition to the roots, also other tissues of the plant; the term is also applied to the association of fungi with mosses and liverworts, which do not form true roots. In view of the fact that the relation between the fungus and the host plant is not the same in different cases, it is best to apply the term *symbiosis* for designating the living together of dissimilar organisms, as defined by deBary in 1879<sup>1</sup>, and not an association which is of mutual benefit to both participants, as is usually done.

Pfeffer<sup>2</sup> was the first to call attention to the possible symbiotic action between the roots of plants and fungi. Frank<sup>3</sup> divided the mycorrhiza into two groups: 1. *Ectotrophic mycorrhiza*, when the fungus produces an external investment of the root, in the form of a crown of hyphae, without penetrating into cells other than those of the epidermis; there is an extensive intercellular development between the cortical cells of the root; this is especially characteristic of forest trees. Frank believed that *Pinus sylvestris*, for example, does not reach maturity on good pine soils, unless a specific fungus necessary for the formation of the mycor-

<sup>1</sup> Magrou, J. Proc. First. Intern. Congr. Soil Sci. 3: 72-91. 1928; Rayner, M. C. Ibid. 317-324. 1928.

<sup>2</sup> Pfeffer, W. Landw. Jahrb., 6: 969-998. 1877.

<sup>3</sup> Frank, B. Ber. deut. bot. Gesell., 3: 125, 143. 1885; 5: 395-408. 1887; 9: 244. 1891; 10: 577-583. 1892; Forstwiss. Centrbl. 16: 185-190. 1894.

rhiza is present. 2. *Endotrophic mycorrhiza*, in which the hyphae of the fungus penetrate to the inner parts of the roots, into definite root layers and into the cells, and have little connection with the mycelium in the soil; this is true of the Orchidaceae, Ericaceae and Eparidaceae; however, it is now known also for many other plants. Root hairs were frequently absent in ectotrophic mycorrhiza and were replaced by hyphae of fungi. Frank formulated the theory that these absorb the mineral salts and organic nitrogen compounds for the plant, whereas the latter supplies the fungus with synthesized carbohydrates. In the case of the endotrophic mycorrhiza the plant obtains nitrogen from the fungus in the process of digestion of the mycelium.

Since Frank's earlier work, many transition forms between the two types of mycorrhiza have been recorded, some of which can be described as ecto-endotrophic types. Melin<sup>4</sup> described three types of mycorrhiza formations on the Scotch pine: 1. *Forked mycorrhiza*, best developed in the presence of an abundant layer of raw-humus; it is golden-brown to black in color. 2. *Tuber mycorrhiza*, pale at first, later becoming gray to brownish-gray in color. 3. *Simple mycorrhiza* or the unbranched form characteristically found on the pine in the woodland and pine-heath soils. This may be a young stage of the forked or tuber type, or it may be a result of conditions unfavorable for the optimum growth of the fungus. Melin also recognized *pseudo-mycorrhiza*, which are endotrophic in nature but are not comparable to the true endotrophic forms in orchids; the hyphae are not digested and the fungus is largely parasitic.

Another type of mycorrhiza has been recognized by Peyronel,<sup>49</sup> which seems to have no definite relation to seed germination. The mycelium passes through the outer layers of the root, forming haustoria-like endings (arbuscles). The mycelium forms swollen terminal or intercalary portions (vesicles). The arbuscles finally break up, leaving small round oil-bearing bodies. The systematic position of this fungus is discussed later (p. 264). These mycorrhiza are very frequent in herbaceous plants.

<sup>4</sup> Melin, E. Bot. Tidskr. 16: H. 2. 1922; 17: H. 4. 1923; Bot. Notiser, 1924. p. 69; Mykolog. Unters. u. Ber. by Falck. 1923; Untersuchungen über die Bedeutung der Baummykorrhiza. G. Fischer, Jena. 1925. A detailed review of the earlier literature on mycorrhiza is given by Bernard, N. Ann. Sci. Nat. Bot. (9), 9: 1-186. 1909; Gallaud, I. Rev. gen. Bot., 17. 1905; Janse, J. M. Ann. Jard. Bot. Buitenzorg., 14. 1897; Burgeff. Handw. Naturw. G. Fischer. Jena. 9. 1913; Rayner, M. C. Mycorrhiza. London. 1927.

Another type of plant-fungus association is found in the case of certain forms of rye-grass (*Lolium*). A stratum of fungal hyphae is found between the seed coat and the aleurone layer. On germination of the seed, the fungus attacks the growing part of the stem and develops with it. It then penetrates the ovary and thus develops in the seed. *Lolium* also forms a mycorrhiza in association with the fungus of the Phycomycete type; there seems to be no definite connection between the two fungi.

The penetration of the fungus hyphae into the roots of the plant, which results in the formation of an endotrophic mycorrhiza, can take place (as described for *Lolium temulentum*<sup>5</sup>) in two different ways: 1. If the hypha reaches the root hair, it may penetrate through its wall and grow through the cavity until it reaches the corresponding epidermal cell; sometimes the hypha may twist round the hair in one or two close loops before penetrating. 2. The filament may enter the root by piercing the epidermis directly. Having reached the interior of the root, the filament twists in a loose spiral-like fashion in the lumen of the epidermal cell. Growth takes place at right angles to the root surface and the next layer of root cells is infected before there is a considerable horizontal extension of the mycelium in the root. The hyphae in the epidermis and in the outer cortical layers are intracellular in nature, but, with horizontal spreading of the mycelium in the second root layer, branching takes place, some of the branches penetrating the cell wall into the intercellular spaces. Vesicles are formed in the second region; they are looked upon as arrested sporangial developments or as spore bearing bodies which may function as temporary reserve organs during the early stages of the invasion of the fungus. Arbuscles and sporangioles are formed in clusters in the third region, the middle cortex.

*Plants forming mycorrhiza.* A large number of plants, including perennials and annuals, are capable of forming mycorrhiza with different fungi. The association may either be constant or not. The presence of the fungus or its absence frequently depends largely upon external conditions. Wild plants and fruit trees form mycorrhiza more readily than cultivated plants. Some fungi can attack a large number of plants, whereas some plants can form mycorrhiza with different fungi. This phenomenon is especially highly developed in certain cases, as in the orchids and heath plants (*Ericaceae*); in many instances, there is no direct specificity between the plant and the infecting fungus. Stahl<sup>6</sup>

<sup>5</sup> McLennan, E. I. Ann. Bot., 40: 43-68. 1926.

<sup>6</sup> Stahl, E. Jahrb. wiss. Bot., 34: 539-668. 1900; Jahrb. wiss. Bot., 49: 579-615. 1911.

and others demonstrated that most higher plants, with the exception of submerged water plants and certain specific large families of plants (*Cruciferae*, *Cyperaceae* and *Polypodiaceae*), possess always or occasionally mycorrhiza formations. In the case of the *Compositae*, for example, as many as 85 per cent of the plants examined were found<sup>7</sup> to produce endotrophic mycorrhiza. Stahl suggested that plants with rapid transpiration can absorb their mineral food material without the fungi, whereas plants with weak transpiration can obtain a sufficient supply of minerals only by the assistance of the symbiotic fungi. Stahl's theories, however, were not based on experimental evidence.

Bernard observed a series of transition stages in the degree of dependence of the plant upon the fungus. In case of a few species of orchids, as *Bletitia*, the seeds germinate but are unable to form roots, without infection; in the majority of other orchids, the embryo is arrested at a much earlier stage. The degree of specificity between plant and fungus varies, as does the resistance of the plant to the fungus invasion. It was demonstrated experimentally that symbiosis between the plant and fungus is obligate. The results obtained by practical orchid growers confirmed these facts. Bernard and Magrou<sup>8</sup> developed a theory of tuber formation, in the evolution of plants, based on the symbiotic action of the plants with the fungi. The symbiosis in potatoes and in *Orobis tuberosus* germinating from seed results in the formation of tubers out of the buds at the base of the stem. When the plants are kept from becoming infected with the symbiotic fungi or do not establish the usual symbiotic relation, without otherwise changing the conditions of growth, the same buds change into thin stems, without the formation of tubers (No. 116, Pl. XII). This theory is still in the theoretical stage, since the evidence is incomplete.

Under natural conditions, orchid seeds contain fungus hyphae in the cells of the root cortex. Bernard believed that the difficulty in germinating orchid seeds is due to a need for fungus infection. He believed that the fungus raises the concentration of the cell sap of the plant. By increasing the percentage of organic constituents in the medium, seeds can be germinated without the intervention of the fungus, especially in the case of *Cattleya*. According to Knudson,<sup>9</sup> the fungus brings about

<sup>7</sup> McDougall, W. B. and Glasgow, O. E. Amer. J. Bot. 16: 225-228. 1929.

<sup>8</sup> Magrou, J. Ann. Sci. Nat. (10), 3: 181-275. 1921; 7: 725-278. 1925.

<sup>9</sup> Knudson, L. Bot. Gaz. 73: 2. 1922; 77: 212. 1924; see Ramsbottom, J. Proc. intern. Congr. Plant Sci. Ithaca, 2: 1676. 1926.

the production of sugar in the medium which exerts a favorable effect on germination.

A relation between plant and fungus resembling that found in orchids was also observed in Ericaceae, namely a complete dependence of the seedling plant upon infection by the endophyte at a critical stage, differing, however, in the mode of infection.<sup>10</sup> In the absence of infection, root formation is arrested and growth is finally inhibited. Infection of the primary root takes place soon after germination of the seed, which is infected while still in the ovary. The dissolution of the fungus takes place here as well. The fungus, though present largely in the roots, is present, in an attenuated form, throughout the plant, in the stem, leaves and fruit. The formation by *Arbutus unedo* of root tubercles, which are arrested secondary and successive laterals of the season's growth, is due to the invasion by a fungus, which at first acts ectotrophically, then as an endotroph of the peripheral cells.<sup>11</sup> For the development of various for-

<sup>10</sup> Rayner, M. C. The New Phytol., 10: 227-240. 1911; Ann. Bot., 29: 97-133; 1915; Brit. Mycol. Soc., 8: 61-66. 1922.

<sup>11</sup> Rivett, M. F. Ann. Bot., 38: 661-677. 1924.

## PLATE XII

### MYCORRHIZA FUNGI

111. Apparatus for rooting cuttings under controlled conditions: *w*, cotton; *c*, cutting; *s*, sand; *r*, glass rod; *h*, rain water; *p*, potash tube (from Rayner).

112. Vessel for study of mycorrhiza formation in pure culture (from Melin).

113. Hyphae of *Tricholoma flavobrunnea*, grown in pure culture in symbiosis with birch tree,  $\times 50$  (from Melin).

114. Oblong section of hyphae radiating from mycorrhiza-root,  $\times 500$  (from Melin).

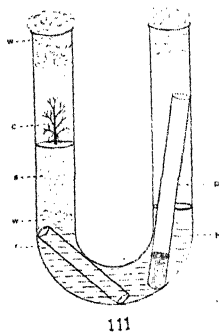
115. Beginning of infection of epidermis of young wheat root by phycomycoid endophyte; *p*, points of entrance of mycelium into the root; attention is called to the growth of the mycelium between the cells,  $\times 130$  (from Peyronel).

116. Stages of evolution, showing the process of tuber formation as a result of symbiosis: *A*, *Solanum tuberosum*; *B*, *Orobis tuberosus*; *C*, *Ficaria ranunculoides*; *D*, plantlet of *Blechna hyacinthina* inoculated with attenuated *Rhizoctonia repens*; *E*, plantlet of *Bl. hyacinthina* inoculated with an active *Rh. repens*; *F*, embryo tuber of *Catleya*; *t* = tubers (after Bernard and Magrou).

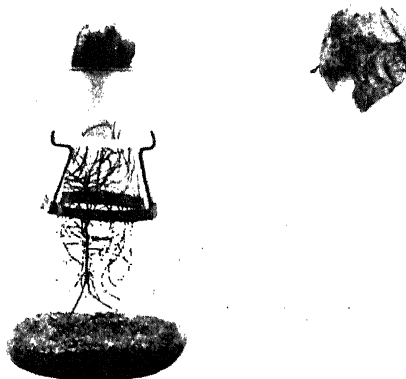
117. Longitudinal section of a potato root, showing an early stage of fungus infection; *m*, coiled mycelium; *n*, cellular nuclei; *n'*, fungus nuclei (from Magrou).

118. Two infected cells of a potato root, the lower cell showing large bodies resulting from disintegration by phagocytosis and the upper cell showing non-disintegrated mycelium which attacks the cell (from Magrou).

119. Mycorrhiza cells from young root of seedling of *Calluna vulgaris* showing, at right, "clumping" at early stage of digestion and, at left, digestion process (from Rayner).



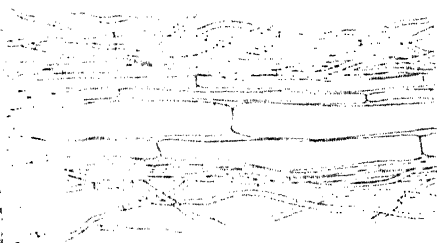
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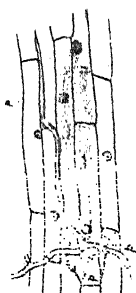
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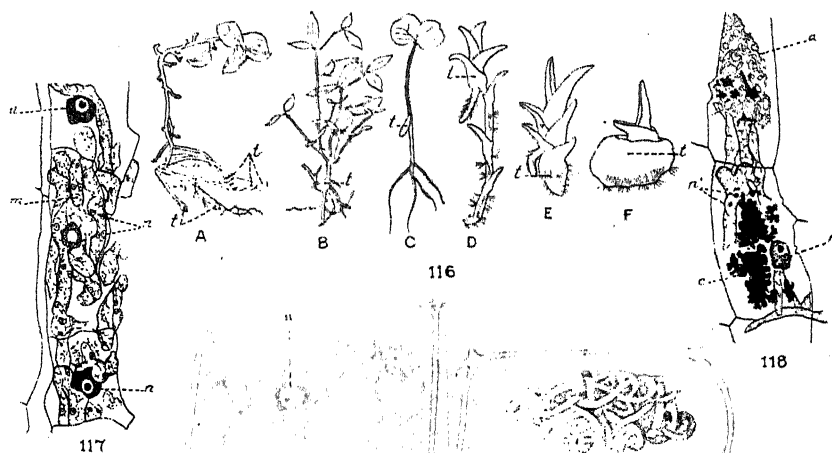
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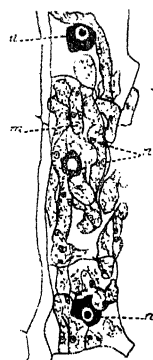
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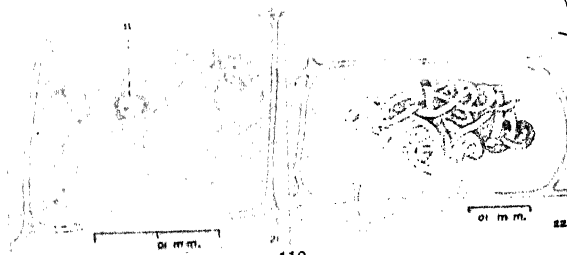
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est trees growing in raw-humus soils (p. 689), such as firs and spruces, mycorrhiza are absolutely essential;<sup>12</sup> without them the plant development will not be normal. A large number of other annual and perennial plants were also found<sup>13</sup> to form mycorrhiza; here are included various plants among the *Graminaceae*, *Araceae*, *Liliaceae*, *Amaryllidaceae*, *Iridaceae*, *Juglandaceae*, *Urticaceae*, *Lauraceae*, *Polygonaceae*, *Chenopodiaceae*, *Portulacaceae*, *Violaceae*, *Cruciferae*, *Ranunculaceae*, *Saxifragaceae*, *Rosaceae*, *Umbelliferae*, *Geraniaceae*, *Rutaceae*, *Primulaceae*, *Solanaceae*, *Scrophulariaceae*, *Compositae*, etc. The roots of various legumes are extensively invaded by a characteristic fungus belonging to the mycorrhiza type. The fungus is found in the primary cortex and forms a coarse, nonseptate mycelium in the roots and sends out haustoria into the deeper cells, often filling more than half of the lumen of the cell. The fungus is well distributed in the soil, and can infect most legumes and various nonleguminous plants.<sup>13a</sup>

In addition to these plants, a number of Bryophytes (liverworts<sup>14</sup>) and Pteridophytes seem to form an association with fungus mycelium in their tissues similar to the mycorrhiza. Various salt marsh plants, such as *Plantago coronopus* and *Glyceria maritima*, are capable of forming endotrophic mycorrhiza.<sup>15</sup>

Among the Ericaceae, the genus *Vaccinium* occupies a special place due to its economic importance. The mycorrhiza of various species of *Vaccinium* were described in 1887 by Frank.<sup>15</sup> Stahl stated in 1900 that roots of *Vaccinium myrtillus* remained free from fungus infection when plants are grown in sterilized soils, even from untreated seeds. Under normal conditions, however, these plants possess a well developed mycorrhiza of the ericoid type.<sup>16</sup> According to Rayner, this must therefore involve infection from the soil and preclude infection from mycelium present in the seed coat. Later investigations of Rayner<sup>17</sup> demonstrated that the seeds of *Vaccinium oxycoccus* and *V. macrocarpum* are infected by their respective endophytes while still present in the fruit, this infection being more deep seated than that of *Calluna*. Ternetz<sup>18</sup>

<sup>12</sup> Melin, E. Mykol. Unters. u. Ber., 2: 73-335. 1923.

<sup>13</sup> Peyronel, B. Riv. Biol., 5: 463-485. 1923; 6: 17-53. 1924.

<sup>13a</sup> Jones, F. R. Jour. Agr. Res. 29: 459-470. 1924.

<sup>14</sup> Ridler, W. F. F. Ann. Bot. 36: 193-208. 1922; 37: 483-488. 1923; Magrou, J. Ann. Sci. Nat. Bot. (10) 7: 725. 1925.

<sup>15</sup> Mason, E. New Phytol. 27: 193-195. 1928.

<sup>16</sup> See Coville, F. V. Bur. Pl. Ind. Bul. 193, 1911.

<sup>17</sup> Rayner, M. C. Ann. Bot. 169: 55-70. 1929; see also Doak, K.D. Phytopath. 18: 148. 1928.

<sup>18</sup> Ternetz, C. Jahrb. wiss. Bot. 44: 353-408. 1907.

was the first to isolate the fungus *Phoma radidis* from *Vaccinium* and claimed that this organism is capable of fixing atmospheric nitrogen. These claims were later substantiated by Rayner.

*Ectotrophic mycorrhiza.* These mycorrhiza occur abundantly on woody plants. They usually form a dense mass of hyphae around the apical portions of the infected roots. Root hair development is prevented and short, corraloid branches are formed instead. Not all the roots are attacked; only those that develop in the layers of decomposing leaf material, i.e. in the mass of organic matter. Within the root tissues there is found a continuous network of hyphae which separate the individual cells of the epidermal and cortical layers; the fine hyphae composing the network are continuous with those of the external mantle and frequently penetrate the epidermal cells.

The fungus infection leads to the shortening of the roots and their development into mycorrhiza. The short roots are branched in a characteristic manner, usually forklike. As pointed out above, Melin recognized several types of tree mycorrhiza in pine trees. The forked mycorrhiza is well developed in raw-humus forest soils; growth of root hairs is arrested and the dichotomous branching leads to the formation of a dense tuft or "witches broom." The tuber mycorrhiza presents the appearance of small tubers, variable in size and frequently grown together; it results from the merging of a cluster of dichotomously forked roots together; this type is usually associated with species of *Boletus*. The spruce possesses two types of mycorrhiza: racemose and simple. The structure and development of the fungus mantle varies depending upon soil conditions and the nature of the infecting organism. Some of the ectotrophic formations may be accompanied also by endotrophic infection of the cortical cells. If the seedlings are weak or the mycelium especially vigorous, the fungi may actually become parasitic upon the plant; if the seedlings are healthy and the mycelium well developed, equilibrium is established described by Melin as mutualism. The nature of ectotrophic mycorrhiza of *Alnus* was further described by Masui.<sup>19</sup>

*Endotrophic mycorrhiza.* In the case of many plants, especially the *Orchidaceae* and *Ericaceae*, fungal hyphae enter the cortex of the root from the soil, leading at first a parasitic existence on the outer root layers; later, especially in well nourished cells, the hyphae are digested by the host leaving round bodies as residues. Although the fungus does

<sup>19</sup> Masui, K. Mem. Coll. Sci. Kyoto Imp. Univ. S. B., 2: 161-187, 189-209. 1926.

not sporulate within the tissues of the healthy host plant, it can be isolated and grown in culture. Some orchids (*Cattleya*) are rootless and are free from the fungus during a part of the year. Others (*Vanda*) have permanently infected roots. The seeds of orchids are small and do not germinate in the absence of the fungus. Infection takes place in the process of seed germination. In the case of some orchids at least (*Loelia*, *Cattleya*), the seeds will germinate without the fungus, providing fructose, maltose, or other sugar is present.<sup>20</sup> The failure of the orchid seed to germinate without the fungus is ascribed to the low content of reserve material in the seed, making it dependent upon outside sources for organic food; under natural conditions, the fungus is believed to make the organic matter of the soil available to the seed. The following conditions were finally established for the successful germination of orchid seeds: a suitable solution of mineral salts, a satisfactory sugar supply, and a favorable pH (4.8 to 5.2). All infection seems to take place from the soil. The fungi are to a certain extent specialized upon the different orchids.

*Calluna vulgaris* is associated with a fungus throughout its development, the mycelium occurring not only in the roots but also in the stems, leaves, flowers and fruit.<sup>21</sup> When the seeds germinate, fungus hyphae present in the seed coat infect the seedling, which results in its normal development. The presence of a fungus (*Armillaria mellea*) is also quite important for a normal development of the non-chlorophyll-bearing orchid *Gastrodia elata*. Without infection, the resting stage of the orchid, in the form of a tuber, becomes smaller every year. On infection by the fungus, however, it produces a flowering shoot. A certain balance is established between the tuber and the fungus, the fungus mycelium passing the nutrients from the decomposing tree to the orchid. The orchid then becomes a parasite upon the fungus.

Gallaud classified the endotrophic mycorrhiza into four types, based upon the intra- or inter-cellular localization of the mycelium and formation of arbuscles or sporangioles: 1. *Arum maculatum* series, 2. *Paris quadrifolia* series, 3. Hepatic series, and 4. Orchid series. These groups had no taxonomic significance. Bernard regarded the root fungi of

<sup>20</sup> Knudson, L. Bot. Gaz. 73: 1-25. 1922; 77: 212-219; 79: 345-379. 1925; New Phytol. 26: 328-336. 1927; 28: 369-376. 1929. La Garde, R. V. Ann. Mo. Bot. Gard. 16: 499-514. 1929; Quednow, K. G. Bot. Archiv. 30: 51-108. 1930; see Fuchs, A. and Ziegenspeck, H. Bot. Arch. 9: 361-464. 1925; 16: 360-413. 1926.

<sup>21</sup> Rayner, C. New Phytol. 28: 377-385. 1929.

orchids as parasites, attacking the embryo in its early development, then persisting in the plant.

*Organisms responsible for mycorrhiza formation.* Various species of fungi have been reported to be able to live symbiotically with higher plants. In the case of ectotrophic mycorrhiza, mostly higher fungi have been found. In some cases, certain Phycomycetes, including the Mucorales, have been reported; in a few instances, Actinomyces and Penicillium were believed to be involved. According to Hagem, certain Mucorales, especially *Zygorhynchus mölleri*, *Mucor ramannianus*, certain species of Absidia (*A. orchidis*) and a green Penicillium may form mycorrhiza with forest trees. Möller<sup>22</sup> also suggested the possibility of formation of mycorrhiza by *Zygorhynchus*. All these investigations are open to criticism, however, since no experimental formation of mycorrhiza was attempted.

MacDougal and Peyronel<sup>23</sup> came to the conclusion that fungi belonging to the Oomycetes, Gasteromycetes, Hymenomycetes and Pyrenomycetes can form mycorrhiza, but in these cases as well no experimental evidence was supplied. One must discriminate carefully between attributions based merely on isolations of species of fungi from mycorrhiza-bearing plants and those in which the mycorrhiza has been produced again experimentally by pure cultures of the organism isolated from mycorrhiza. Positive proof of identity can only be supplied by inoculation of a seedling growing in pure culture, under controlled conditions, with subsequent production of mycorrhiza. In the extensive literature on mycorrhiza formation, very few attempts which have been made to isolate the causative organism and cultivate it in pure culture, or to produce mycorrhiza artificially, have proved successful. This has been accomplished in the case of various orchids,<sup>24</sup> in *Calluna vulgaris*,<sup>25</sup> and especially by Melin for various pine trees. Bernard classified the fungus of orchids with *Rhizoctonia*, on the basis of sclerotia formation. Burgeff, however, later termed this organism Orcheomyces. The organism isolated from *Calluna vulgaris* was placed by Rayner in the genus *Phoma* among the Sphaeropsidales.

<sup>22</sup> Möller, A. Ztschr. Forst. u. Jagdwesen. 1903, H. 5-6; Hagem, O. 1907 (p. 221).

<sup>23</sup> MacDougal, D. T. Ann. Bot., 13: 1-48. 1899; Peyronel, B. Riv. Biol. 5: 463-485. 1923; 6: 17-53. 1924.

<sup>24</sup> Bernard, 1909 (p. 255); Burgeff, H. Jena. 1911; Die Wurzelpilze der Orchideen. Jena. 1909; Schatz. Beiträge zur Biologie der Mykorrhizen. Inaug. Diss. Jena. 1910.

<sup>25</sup> Rayner, 1927 (p. 255).

The true mycorrhiza fungi of forest trees should be looked for largely among the Hymenomycetes. Various members of the genera *Cortinarius* (between *C. rubripes*, on the one hand, and *Quercus rubra*, *Picea rubra*, or *Acer saccharum* on the other), *Russula* (between *R. emetica* and *Quercus rubra*), *Tricholoma* (*T. speciosus* or *T. transmigrans* and *Quercus nigra*), *Armillaria* (*A. mellea* and the orchid *Gastrodia elata*), *Boletus* (*Boletus* species and pine trees) were found to be the causative agents of mycorrhiza formations.<sup>26</sup> Mycorrhiza formation by Gasteromycetes is still questionable; the same is true of the Ascomycetes, although *Rhizoctonia* belongs to this group. A number of Hyphomycetes are capable of entering the roots, where they seem to grow more or less parasitically. Some of the Hymenomycetes, such as *Boletus elegans*, are highly specialized upon certain host plants (larch) whereas some are less specialized, such as *Amanita muscaria*, and are capable of forming mycorrhiza with various plants, such as pine, spruce, larch and birch.

*Rhizopogon rubescens* was reported as the mycorrhizal fungus on *Pinus sylvestris* in the United States. It was suggested that it is an important soil organism for nursery stock. This organism is universally present in sandy soils under pines.<sup>27</sup>

Christoph<sup>28</sup> obtained from *Calluna vulgaris* a *Cephalosporium* and claimed to have produced the mycorrhiza synthetically; however, his experimental work is open to criticism.<sup>29</sup> Huber<sup>30</sup> isolated from *Liparis laeselii* a *Rhizoctonia* (*R. repens*). A species of *Rhizoctonia* (*R. apocyanacearum*) was also isolated from *Vinca minor* by Detemar.<sup>31</sup> The fungus grows in the root in all directions, except the endodermis, both inter- and intra-cellularly. When the fungus penetrates into the cells, the starch content even of the neighboring cells begins to decrease. Infection of the plant can take place all the year around, but especially in March to May.

The organism studied by Detemar forms a special type of endotrophic mycorrhiza, termed "plasmoptico-mycorrhiza." This is due to the fact

<sup>26</sup> Kauffman, C. H. Bot. Gaz. 42: 208-214. 1906; Pennington, L. H. Rpt. Mich. Acad. Sci. 10: 47. 1910; Rommel, L. G. Svensk. Bot. Tidskr., 15: 204-213. 1921; Melin, E. Karst. Svensk. Bot. Tidskr., 15: 192-203. 1921; Jour. Ecol., 9: 254-257. 1922; see also p. 265.

<sup>27</sup> Baxter, D. V. Mich. Acad. Arts Lett. 9: 509-516. 1928; Zeller, S. M. and Dodge, C. W. Mo. Bot. Gard. 5: 1-36. 1918.

<sup>28</sup> Christoph, H. Beih. Bot. Centrbl. 38: H. 2, 115. 1921.

<sup>29</sup> Rayner, 1922 (p. 258).

<sup>30</sup> Huber, B. Sitz. Ber. Ak. Wiss. Wien. (1), 130 (Ref. Detemar).

<sup>31</sup> Detemar, K. Flora, 116: 406-456. 1923.

that some of the fungus cells undergo plasmogamy as a result of certain antibody formation by the plants following the infection by the foreign fungus. This process is a step towards the assimilation of the fungus plasma by the plant. *Vinca minor* is obligately mycotrophic, two-thirds of the root system being infected with the fungus. The fungus can be readily grown on peptone and starch media.

According to Peyronel<sup>32</sup> the organisms responsible in most instances for the formation of endotrophic mycorrhiza in phanerogams, except in the case of the Orchidaceae, form branched or swollen haustoria and are considered to be true Phycomycetes (No. 115, Plate XII). Theoretical considerations led him to consider these organisms as belonging to a primitive group, which gave rise to the two divergent series, the Phycomycetes and Mycomycetes. That group would also include *Endogone*, which may represent one of the phases of the biological cycles of the mycorrhizal endophyte. The vesicles of the phycomycetoid endophyte are very likely provisional stores of reserve material. Some of them may change afterward into apogamous oospores or into sporangia, which remain latent for a long time and mature their spores only when environmental conditions are favorable. By means of these spores the organism spreads through the soil. The mycelium forms a network in the soil, surrounding the root system of the plant and growing from one plant to another. The phycomycete leads both a saprophytic and endophytic existence. The endophytes of orchids were believed to have no similarity to these endophytic phycomycetes, but were considered to be true Mycomycetes, perhaps Basidiomycetes. In addition to the endophytic phycomycete, most plants show that the mycelium of the latter is overgrown with the endophyte of the orchid mycorrhiza fungus. This form belongs to *Rhizoctonia solani* Kühn or *Moniliopsis aderholdi* Rühl, among the primitive basidiomycetes (*Hypochnus* group). Peyronel also found, in addition to these two endophytes, a certain number of saprophytic fungi living at the expense of weak or dead tissues in nearly all plants studied; these fungi belong to the genera *Pythium*, *Fusarium*, *Dydymopsis* and *Rhizomyxa*. *Asterocystis radialis* was found not only on the dying roots, but also regularly on those having a normal appearance, and was also classed as a mycorrhiza fungus. Out of 150 species of phanerogams growing in different localities and under different environmental conditions, Peyronel found the *Rhizoctonia* type in 135. The phycomycetic mycelium, with which the *Rhizoctonia* is usually asso-

<sup>32</sup> Peyronel, 1924 (p. 262).

ciated, develops before the Rhizoctonia, thus facilitating the penetration of the latter and making the host plant more receptive.

Melin<sup>33</sup> differentiated between true mycorrhiza and pseudo-mycorrhiza in forest trees. The fungi of the former belong to the Hymenomycetes, including various species of *Boletus*, comprising *B. luteus*, *B. variegatus*, *B. granulatus*, *B. lactius*, *Amanita* (*A. muscaria*), *Tricholoma*, *Lactarius* (*L. deli*), *Cortinarius* (*C. muscosus*), *Russula* (*R. fragilis*), some being more specialized than others. These form mycorrhiza with pine trees, birches, poplars, and other forest trees. Pseudo-mycorrhizas are formed by a number of common soil fungi (*Mucor*, *Verticillium*), which can penetrate the roots, when they are not infected by the true mycorrhiza; *Mycelium radices atrovirens* (probably *Cladosporium humifaciens*) belongs to the second group. Penicillia are indifferent to the roots. There is a competition in forest soils between the different fungi, in attacking the roots of the trees. The true mycorrhiza formers, when present, will enter the root first; these grow best at pH 5.0 and only poorly at pH 7.0 and pH 3.5;<sup>34</sup> this has an important bearing upon mycorrhiza formation. In neutral or slightly acid soils, these fungi are not virulent and either cannot form at all or form only with difficulty endo- and ecto-trophic mycorrhiza. The most abundant mycorrhiza of evergreen trees are found in soils of pH 4.0 to 5.0. This is the reason why mycorrhiza are formed in acid, raw-humus soils, and not in neutral or alkaline soils. Phosphatides greatly favor fungus development. *Rhizoctonia silvestris* and *Mycelium radices atrovirens* grow well in neutral or slightly alkaline media. When the reaction of the soil is near neutrality, pseudo-mycorrhiza will be formed. Melin succeeded in isolating a series of fungi and demonstrated that the same types of mycorrhiza are produced in pure culture as in the soil; these facts established experimentally allow a better insight into the rôle of mycorrhiza in plant nutrition and plant distribution.

Many of the mycorrhiza forming fungi are specially adapted to certain trees; some are less specific, while others grow without association with the living tree. When a forest is removed, the obligate mycorrhizal fungi will disappear from the soil and reappear again only when the new crop begins to develop; the spores of these fungi do not germinate on artificial media, while the mycelium and the fruiting bodies do not develop when not connected with living tree roots.

<sup>33</sup> Melin, E. Svensk. Bot. Tidskr., 17: 479-520. 1923.

<sup>34</sup> Melin, E. Bot. Notiser, 1924, 38-48; Svensk. Bot. Tidskr., 16. 1922.



For determining whether a certain organism forms a mycorrhiza with a given plant, sand or forest humus can be used as a substrate. In the first case, 150-gm. portions of pure, washed, dry sand are placed in 300-cc. Erlenmeyer flasks. Forty-nine cubic centimeters of the following medium are then added to each flask:

NH <sub>4</sub> Cl.....	0.50 gram	CaCl <sub>2</sub> .....	0.10 gram
		NaCl.....	0.10 gram
KNO <sub>3</sub> .....	0.95 gram	MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.30 gram
Glucose.....	0.50 gram	FeCl <sub>3</sub> .....	0.01 gram
KH <sub>2</sub> PO <sub>4</sub> .....	1.00 gram	Distilled water.....	1000 cc.

The flasks are plugged with cotton and sterilized in flowing steam for 25 minutes on 3 consecutive days. The humus medium is prepared by saturating with water



FIG. 9. Seedlings of *Picea abies*, three years old, in pure culture, with nucleic acid supplied as a source of nitrogen. A, seedling without fungus infection; B, seedling infected with the mycorrhizal fungus, *Mycelium radialis abietis*. About  $\frac{1}{4}$  natural size (from Melin).

70 grams of mixed forest humus material (upper part of slightly decomposed layer). The flasks are sterilized in steam three times, and washed twice with sterile distilled water by adding 50 cc. of water to each flask, shaking, and allowing it to rest for 24 hours, then pouring off the excess of water. This process is repeated. The contaminated cultures (examined after 14 days) are discarded.

The seeds are sterilized by moistening with water, keeping one minute in 1:1000 mercury bichloride solution, then washing in sterile water. They are then allowed to germinate upon sterile agar (1.2 per cent agar in distilled water), the individual seeds being removed from one another so that the infected seeds do not infect the sterile ones. After 10 to 15 days, the germinated seeds remaining sterile are transferred by means of a sterile platinum needle to the flasks. Contaminated flasks (as shown microscopically and culturally) are discarded. The flasks are then inoculated with the cultures of the fungi in question (fig. 9).

For the study of the rôle of fungi in the germination of seed, Knudson used a different method: 1.5 per cent of agar was added to one of the following nutrient solutions:

	PFEFFER'S SOLUTION	SOLUTION B
$\text{Ca}(\text{NO}_3)_2$ .....	1.0 gram	1.0 gram
$\text{K}_2\text{HPO}_4$ .....	0.25 gram	0.25 gram
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .....	0.25 gram	0.25 gram
$\text{KNO}_3$ .....	0.25 gram	
$\text{KCl}$ .....	0.12 gram	
$\text{FeCl}_3$ .....	0.01 gram	
$\text{Fe}_2(\text{PO}_4)_3$ .....		0.05 gram
$(\text{NH}_4)_2\text{SO}_4$ .....		0.50 gram
Distilled water.....	1000 cc.	1000 cc.

The medium is placed in tubes, 180 mm. by 18 mm.; the tubes are plugged with cotton, autoclaved and slanted; the cotton plug is covered with a cap to prevent contamination. The seeds are sterilized in calcium hypochlorite solution (10 grams in 140 cc. distilled water, shaking well and filtering), by covering them several times in a test tube with the clear solution, for about 15 minutes. By means of a sterile platinum needle the seeds are transferred upon the surface of the sterile agar slope. The cultures are kept in moist chambers in the greenhouse shaded by cheesecloth from direct sunlight.

*Rôle of mycorrhiza in the nutrition of plants.* Various theories have been suggested by different investigators in an attempt to explain the rôle of the fungi in the nutrition of plants forming mycorrhiza.

1. Symbiotic action between the hyphae of the fungus and the root cells of the higher plants. Frank's theory was that in ectotrophic mycorrhiza the mycelial mantle takes the place and functions of the root hairs; the fungus absorbs the mineral salts and nitrogenous materials from the soil organic matter, while the host plant supplies the fungus with carbohydrates. At first, Frank claimed a similar rôle for the endotrophic mycorrhiza, but since the endophyte has little connection with the exterior of the plant, the above theory was modified to state that the plant procures the nitrogen by digesting the fungus. Stahl also suggested that there is a relation between the low transpiring powers of the plant and the presence of the mycorrhiza fungi in the roots, the latter supplying the former with mineral salts obtained from the soil.<sup>35</sup>

<sup>35</sup> See also Shibata, K. *Jahrb. wiss. Bot.*, **37**: 643-684. 1902; Rexhausen, L. *Beitr. Biol. Pflanz.*, **14**: 18-59. 1920; Magrou, J. *Bull. Inst. Past.*, **20**: 169-183, 217-231. 1922.

The symbiotic saprophytism was believed to be a result of the supplemental capacities of two organisms brought into nutritive contact chemotropically.<sup>36</sup> The cells of the higher plants harboring the fungus generally show a decrease in carbohydrate and an increase in protein content. According to Bernard and Burgeff, seed infection of the embryo by the appropriate fungus is essential for the germination of orchid seeds. Knudson<sup>37</sup> suggested, however, that the rôle of the fungus may consist merely in supplying organic matter, since the germination of the orchid seeds depends on an available supply of organic matter. When this is added in the form of certain sugars or plant extracts, germination is made possible without the aid of any fungus. The action of the fungus is ascribed to its production of enzymes which liberate products utilized by the embryo.

2. The fixation of atmospheric nitrogen by the fungi bringing about the formation of mycorrhiza. This subject has received considerable attention<sup>38</sup> because of the important practical application of the phenomenon. Much of the earlier work, however, is largely speculative and is of little practical value. According to Rayner,<sup>39</sup> nitrogen fixation by the endophyte of Ericaceae is demonstrated by the fact that these plants grow on poor soils deficient in available nitrogen and that pure culture seedlings of *Calluna* grow with marked vigor on media free from combined nitrogen. This is especially interesting, since the endophyte belongs to the genus *Phoma* (*P. radialis*), which is known to be capable of fixing atmospheric nitrogen.<sup>40</sup> The amounts of nitrogen fixed by pure cultures, under controlled laboratory conditions were, however, very small. Coville,<sup>41</sup> who found mycorrhiza on the roots of the blueberry, *Vaccinium corymbosum*, favored Ternetz's earlier conclusions concerning the function of mycorrhiza in other species of *Vaccinium*, namely that they are able to utilize gaseous nitrogen; however, these ideas were not based upon experimental evidence. Möller<sup>42</sup> found that ectotrophic mycorrhiza are mostly unable to fix any atmospheric nitrogen. The inability

<sup>36</sup> MacDougal, D. T., 1899 (p. 262).

<sup>37</sup> Knudson, L. Bot. Gaz., **73**: 1-25. 1922; **77**: 212-219. 1924; **79**: 345-379. 1925.

<sup>38</sup> Ternetz, C. Ber. deut. bot. Gesell., **22**: 267. 1904; 1907 (p. 259).

<sup>39</sup> Rayner, M. C. Bot. Gaz., **73**: 226-235. 1922; Jones, W. N. and Smith, M. L. Brit. Jour. Exp. Biol. **6**: 167-189. 1928.

<sup>40</sup> Duggar and Davis, 1916 (p. 252).

<sup>41</sup> Coville, F. V. U. S. Dept. Agr. Bur. Pl. Ind. Bull. **193**, 1910; **334**, 1916; **974**, 1921.

<sup>42</sup> Möller, A. Ber. deut. bot. Gesell., **24**: 230-234. 1906.

to fix nitrogen was also definitely established for the mycorrhiza of forest trees by Melin.<sup>43</sup>

3. The fungi may take an active part in the decomposition of the organic matter in the soil, thus making the nitrogen available for the growth of higher plants. This was first suggested by Frank and later by Shanz and Piemeisel,<sup>44</sup> who made a detailed study of the phenomenon of fungus fairy rings. This is borne out particularly by the fact that mycorrhiza are abundant in soils rich in organic matter; many of the fungi, as the Basidiomycetes, found to be the causative agents of the true mycorrhiza are capable of decomposing difficultly soluble organic substances, such as woody tissues. They need not necessarily absorb the organic matter but merely decompose it, liberating the minerals and nitrogen, which are absorbed by the plant. According to Melin, the rôle of mycorrhiza in the nutrition of trees and other plants growing in peat (raw-humus), and similar soils, consists in obtaining the nutrients from the soil organic matter and supplying it to the plants. In soils with active nitrification, mycorrhiza formation is not essential for the forest trees. Thanks to the mycorrhiza fungi, trees can obtain their nitrogen from the F-layer in forest soil (Hesselmann); a large part of coniferous forests actually owe their existence to mycorrhiza. Constantin and Dufour<sup>45</sup> have shown that the presence of *Boletus granulatus* increases the germination of *Pinus silvestris* and that artificial inoculation of soil causes increased vigor of planted trees.

Burgeff found that plants infected with fungi were favorably influenced in their ability to assimilate carbohydrates from the soil, when the atmosphere was free from CO<sub>2</sub>. It is the general opinion of investigators<sup>46</sup> that the fungus provides the plant with nitrogen. The fungus obtains its nitrogen from the soil organic matter, or it synthesizes its proteins from the inorganic salts of the soil. The plant is known to digest the fungus, just as in the case of insectivorous plants. This intracellular digestion, which is conspicuous in the case of many plants, may be merely a measure of resistance on the part of the vascular plants, but since the products of digestion disappear, it may be assumed that the plants absorb them (Nos. 118, 119, Pl. XIV).

4. A certain amount of evidence has been submitted to indicate that the fungus may sometimes be injurious to the host plant. Gallaud

<sup>43</sup> Melin, 1925 (p. 255).

<sup>44</sup> Shanz, H. L. and Piemeisel, R. L. Jour. Agr. Res., 11: 191-246. 1917.

<sup>45</sup> Constantin, J. and Dufour, L. Ann. Sci. Nat. Bot. 9: 271. 1927.

<sup>46</sup> Weyland, H. Jahrb. Wiss. Bot., 51: 1-80. 1912.

considered the fungus to lead an independent existence in the root tissues, deriving all its food from the host plant and being an "internal saprophyte." Bernard actually considered the fungus to be a parasite which is subsequently checked in its development by the action of the root cells; this confers a certain immunity upon the infected plant, the process being one of phagocytosis. According to Detemer, the root growth of the plant is influenced injuriously by the fungus infection. The fungus decomposes starch readily and its growth is dependent on the presence of the roots of the plant. When the plant is grown in culture, uninfected by the fungus, it will thrive better than when grown under equal conditions in the presence of the fungus.

Boulet<sup>47</sup> found endotrophic mycorrhiza on the roots of various fruit trees and suggested that the fungus appears to live as a parasite on the host; this was believed to have generally a beneficial effect on the host, except when the essential organs of the roots are attacked. The possible parasitism of mycorrhiza (to *Picea*), under certain conditions, has been suggested also by Rexhausen. W. B. McDougal<sup>48</sup> believed that the fungi (belonging to the genus *Cortinarius*) which form ectotrophic mycorrhiza on the roots of *Picea rubra* are of no benefit to the trees concerned; they form no symbiotic associations with the trees, but are instances of parasitism of fungi on the roots of the trees; no harm, however, may be caused by this parasitism. Feeble plants may actually be injured by the mycorrhiza fungi.

Peyronel demonstrated that the phycomycetoid endophyte grows as vigorously under saprophytic conditions as in symbiosis with the plant; it continues its development, after the death of the plant, at the expense of the dead cortical tissues of the roots or organic matter in the soil. Sterilization of soil increases the yield of wheat due to the destruction of the fungi which attack the plant roots (*Rhizoctonia*, *Asterocystis*, *Pythium* and *Fusarium*); these fungi are claimed to form mycorrhiza.<sup>49</sup>

5. An obligate relation between mycorrhiza fungi and the host plant is certain for orchids and ericaceous plants. The mycorrhiza fungi are considered in this case to be not parasitic forms but true mutualistic symbionts. Trees also are not injured, whereas the fungi develop better in association with the host than in pure culture, and are finally digested by the active juices of the plant. Dufrenoy<sup>50</sup> suggested that symbiosis

<sup>47</sup> Boulet, V. Compt. Rend. Acad. Sci., 150: 1190-1192. 1910.

<sup>48</sup> McDougal, W. B. Amer. J. Bot., 1: 51-74. 1914; Jour. Forestry, 20: 255-260. 1922.

<sup>49</sup> Peyronel, B. Boll. R. Staz. Pat. Veg. 6: 348-358. 1926.

<sup>50</sup> Dufrenoy, J. New Phytol., 16: 222-228. 1917.

is a form of parasitism, in which equilibrium exists between the invading power of the fungus and the resisting power of the host; this is profitable to both, so long as it is maintained, but eventually it is a disadvantage or death to either one, if sufficient advantage is gained by the other symbiont. Rayner<sup>51</sup> also considered that an equilibrium becomes established between the attacking mechanism of the fungus and the protective mechanism of the plant. When this equilibrium is broken down, as in the growth of the plant under favorable conditions (*Calluna vulgaris* growing in a calcareous soil), symbiosis changes to parasitism of the fungus upon the plant tissues. Under these conditions, the fungus, which never forms pycnidia in nature when associated with healthy plants, produces these bodies freely in the stomata which invest the roots of the unhealthy seedlings.

A case of symbiosis whereby the higher plant depends completely upon the presence of the endophytic fungus has been recorded for *Gastrodia elata* among the orchids.<sup>52</sup> This non-chlorophyllous plant is parasitic upon the fungus *Armillaria mellea*; the mycelium is digested by the cells of the root, the relation being obligate for the full development of the plant; the fungus itself is parasitic in habit and at first invades the tuber of the orchid. According to Rayner symbiosis in the case of mycorrhiza is considered to imply not a reciprocal relation involving mutual benefit to the participants, but, as defined by de Bary, the living together of dissimilar organisms.

According to Bernard, Magrou and Constantin,<sup>53</sup> the association of perennial plants with soil fungi brings about a condition of permanent symbiosis, non-existent in annual species. They suggested that the processes of tuberization and perennial habit in plants were due to this symbiotic relationship. *Orobanchaceae* is an annual with a well branched subaerial stem not forming any tubers; if the roots are attacked by a fungus, the latter is rapidly digested. However, *Orobanchaceae tuberosus* is a perennial whose flowering branches develop from underground stolons on which tubers are formed; the roots always contain a fungus. When the seeds of *O. tuberosus* are grown on sterilized soil, they give rise to plants similar to those of *O. coccineus*. Similar relations were found in

<sup>51</sup> Rayner, M. C. Jour. Ecol., 9: 60-74. 1921; Brit. Jour. Exp. Biol., 2: 265-292. 1925.

<sup>52</sup> Kusano, S. Ann. Bot., 25: 521-524. 1911; Jour. Coll. Agr. Tokyo, 4: 1-66. 1911.

<sup>53</sup> Magrou, J. Ann. Sci. Nat. Bot. (10) 4: 181. 1921; Proc. First Int. Congr. Soil Sci. 3: 72-91. 1928; Constantin, J. Compt. Rend. Acad. Sci. 174: 1659. 1922.

species of *Mercurialis* and *Solanum*. This phenomenon raises a series of very important problems which remain to be investigated.

The aleurone layer of cereal and grass seeds is believed<sup>54</sup> to be produced by symbiotic fungi. In English rye grass (*Lolium pratense*), for example, the symbiotic fungus is claimed to be present in the aleurone layer and in the endosperm, as well as between the aleurone layer and the seed coat; it forms a distinct mycelium connected with the specific contents of the aleurone cells, which are the products of the fungus. Peklo concluded from cytological studies that the symbiotic fungi of English rye grass, wheat and barley produce the aleurone layer and later penetrate the peripheral cells of the endosperm leaving there the proteins present in their tissues, the so-called gluten. The same type of protein (prolamin) was found in English rye grass seed and in the corresponding symbiotic fungi.

In view of the fact that the mycorrhiza fungus is essential for the development of certain plants, its introduction into the soil, when it is lacking there, may become a prerequisite for the practical growth of the specific plant. It was found, for example, in Western Australia that the only method of raising satisfactory planting stock in a new nursery was to infect the soil with either a light dressing of soil from an old nursery or to transplant seedling pines from an old nursery and hold them a year in nursery lines.<sup>55</sup>

One may well agree with Rayner in her conclusions that the earlier views of Frank and Stahl survived the test of experimental inquiry, although their arguments may not always be acceptable. Recent studies tend to prove that the possession of mycorrhiza is frequently of benefit to the vascular hosts, the nature and extent of the benefit depending upon environmental conditions and the physiology of the specific association.

<sup>54</sup> Peklo, J. Ber. deut. bot. Gesell. 31: 370-384. 1913; Jodidi, S. L. and Peklo, J. Jour. Agr. Res. 38: 69-91. 1929.

<sup>55</sup> Kessel, S. L. Empire Forestry Jour. 6: 70-74. 1927.

## CHAPTER XII

### SOIL ACTINOMYCES

*General considerations.* The actinomyces, or ray fungi, are a group of organisms present in great abundance in nature, particularly in the soil. In the case of some soils, as many as 40 to 60 per cent of the colonies developing on the common agar or gelatin plate are actinomyces. Their abundance and importance in the soil was rather overlooked until the early part of this century, while a systematic study of their occurrence and rôle in the soil appeared only with the work of various investigators begun about 1913. This was due primarily to the fact that these organisms grow only slowly on common nutritive media. They form colonies which are, at early periods of incubation, hardly distinguishable from bacterial colonies. The fine mycelium, which breaks up readily into a number of fragments during the process of staining, has also contributed to the failure of a proper study of the morphology of this group of organisms and their classification. The actinomyces are characterized by uniformity of growth on complex organic media and considerable variability of growth on synthetic media. On nutrient agar, potato agar and other organic media, various strains which are otherwise distinctly different will look alike, as indicated by pigment production, type of mycelium, etc. When synthetic or other specific media are used for purposes of separation, organisms closely related show distinctive cultural differences. Early workers in soil bacteriology grouped all the actinomyces into two or more large classes, characterized by the production of a dark brown pigment on nutrient agar (chromogenic species) or the formation of white aerial mycelium. Hundreds of new species can be described and differentiated by minor cultural differences, such as intensity of pigmentation, rapidity of liquefaction of gelatin or starch, formation of zones on specific media, etc. It is only very recently that an attempt has been made to separate the actinomyces group into definite species and even genera, on the basis of morphological characters.

*General description of the genus Actinomyces.* *Actinomyces* Harz can be readily recognized and easily differentiated from bacteria and from



other fungi. The genus is characterized by the formation of a unicellular mycelium, composed of hyphae, which show true branching, like that of higher fungi. The hyphae are rather long and  $0.5$  to  $0.8\mu$  in diameter (limits  $0.3$  to  $1.2\mu$ ). The mycelium develops either in the substrate or on the surface, in the form of an aerial growth. The aerial mycelium readily breaks up into short fragments, which may look like bacterial rods and also resemble true bacteria in their protoplasmic properties. When examined directly upon the agar or in a hanging drop, or when properly fixed before staining, the aerial mycelium will be found to consist of very fine, characteristic, long or short branching hyphae with the distinct spore bearing hyphae (only the closely related *Mycobacteria* may give a somewhat similar picture).

The reproductive conidia are produced by a simultaneous division of the protoplasm in the sporogenous hyphae, progressing from the tip towards the base. They possess a greater power of resistance than the

### PLATE XIII

#### SOIL ACTINOMYCES

120. Cross section of a young *Actinomyces* "colony" upon agar,  $\times 65$  (from Lieske).

121. Spore germination and consecutive stages of mycelial development of an *Actinomyces* (from Lieske).

122. Young mycelium of an *Actinomyces*, growing 5 days at room temperature,  $\times 400$  (from Lieske).

123. *Actinomyces* (XIII), with a straight type mycelium; long mycelium, little branching: *a* and *b*, long, unbranched hyphae, showing very slight spiral tendency; *c*, chain of spores, *d*, *e*, portions of aerial mycelium showing branching (from Drechsler).

124. *Actinomyces griseus*, with a short mycelium and abundant branching: *a*, *b*, *c*, portions of aerial mycelium; *d*, *e*, spores germinating with one and two germ tubes respectively (from Drechsler).

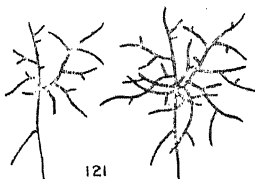
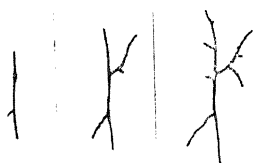
125. *Actinomyces reticuli*, showing the formation of sporogenous hyphae in knot-like groups: *a*, formation of septa in lower sporogenous branches; *d*, formation of secondary branches (from Waksman).

126. *Actinomyces* (IX), showing the narrow closed fist type: *a*, *b*, portions of aerial mycelium, the spiral terminations of which are converted into chains of uninucleated spores; *c*, portion of aerial mycelium with septa in axial filament above insertions of some sporogenous branches; *d*, sporogenous branch; *e*, *f*, germinating spores (from Drechsler).

127. *Actinomyces scabies*, showing open spiral type: *a*, *b*, portions of aerial mycelium, some lateral elements bearing secondary branches, developed successively; *c*, successive stages in development of the sporogenous branch, *c'*<sub>x</sub> and *c'*<sub>y</sub> representing either alternative or probably successive stages (from Drechsler).



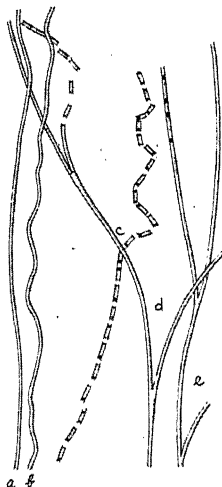
120



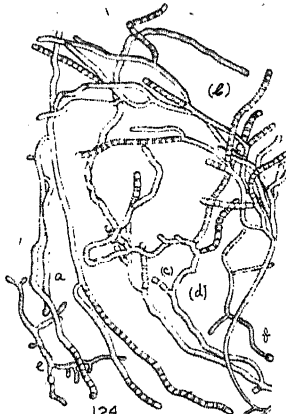
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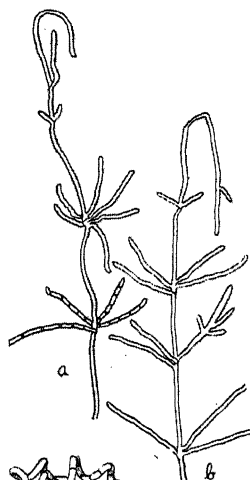
122



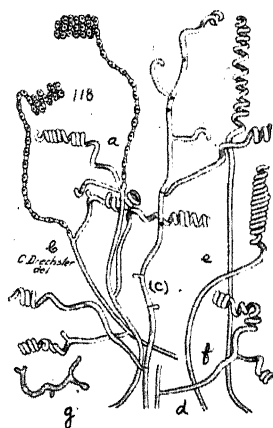
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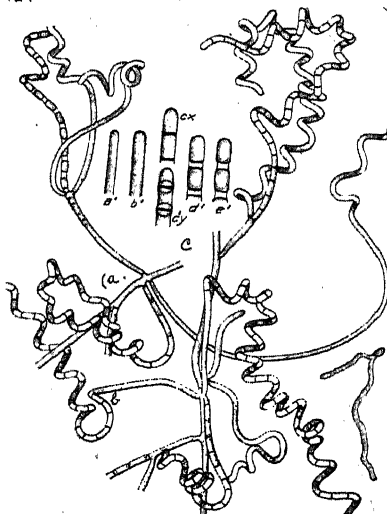
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125



126



127



vegetative hyphae. They resemble bacteria in size, shape and staining properties, are 0.5 to 1.5 microns in diameter, 1 to 2 microns long, oval to rod-shaped. All actinomycetes, particularly in young preparations, are gram-positive. In liquid media, they never cause a turbidity, but grow either on the surface of the medium or in the form of flakes or small colonies throughout the medium; they may sink, especially the flakes, to the bottom of the container or adhere to the glass of the tube. The surface colonies may grow together to form a smooth or wrinkled membrane. The colonies on solid media are tough, leathery, smooth or wrinkled, often growing high above the surface of the medium, and are broken up only when appreciable effort is applied. When transferred to suitable media, almost all the spores germinate, while only a small part of the fragments of mycelium do so. The older the mycelium, the more reduced is the germinating power of the individual fragments.<sup>1</sup>

The aerial mycelium may be white, grey, red, yellow, brown, etc. The aerial hyphae may be short, giving the growth a chalky appearance, long and forming a thick mat over the surface of the growth, or in the form of a fine network. The colonies are often brilliantly colored; some species produce soluble pigments which vary in color and intensity in accordance with the effect of the composition of the medium. Most species are characterized by the production of a sharp peculiar odor characteristic of the soil (earthy odor). All soil species liquefy gelatin; the rapidity of liquefaction depends upon the nature of the organism and previous cultivation. Most of them produce active diastatic enzymes; fewer produce invertase; still fewer produce tyrosinase which enables them to convert the tyrosin of the protein molecule into dark colored melanins.

*Terminology and systematic position.* More names have been applied to this genus than to any other group of microorganisms. Among these, in addition to the proper designation "Actinomycetes," the terms *Leptothrix*, *Cladothrix*, *Oospora*, *Oidium*, *Discomyces*, *Nocardia*, *Streptothrix* and *Microsiphonales* have been used. The terms *Leptothrix* and *Cladothrix* designate two groups of higher bacteria, having nothing in common with the Actinomycetes. *Leptothrix* consists of chains of rod-shaped bacteria surrounded by a thick gelatinous sheath, which gives them the appearance of threads, not forming any true or false branching. *Cladothrix* is similar to *Leptothrix*, forming somewhat thicker threads and possessing false branching. The individual cells may, in the case of

<sup>1</sup> Orskov, J. Investigations into the morphology of the ray fungi. Levin and Munksgaard. Copenhagen. 1923.

both *Leptothrix* and *Cladothrix*, leave the sheath and take the form of swarmers possessing flagella. When a swarmer attaches itself to an old thread and develops into a new thread, false branching arises. *Oospora* and *Oidium* are names of true fungi, which have nothing in common with the actinomycetes. The term *Discomyces* was used in 1878 in an imperfect description of actinomycosis of cattle and was later discarded by its own author. *Nocardia* was used in 1889, long after the term Actinomycetes had been introduced by Harz in an accurate description of the cattle disease; that term is, therefore, without any scientific foundation. A number of investigators still adhere to these terms, especially to that of *Discomyces*<sup>2</sup> and *Nocardia*.<sup>3</sup> The term *Streptothrix*, which has been used very often in designating true actinomycetes, is invalid due to the fact that it was preempted by Corda, in 1839, for a true fungus.

The term *Actinomycetes* was used first by Harz<sup>4</sup> in 1877 in describing the organism causing the actinomycotic disease of cattle. He applied this term, which means "ray-fungus," because of the formation of bottle-shaped and ray-like swellings of the threads of the Actinomycetes granules in the actinomycotic disease of cattle. This term has been accepted in all the leading recent important investigations on this group of organisms, both pathogenic and saprophytic forms.<sup>5</sup> A detailed discussion of the historical development of the terminology of this genus need not be attempted here.<sup>6</sup>

The systematic position of actinomycetes has not been satisfactorily defined. These organisms are classified more often with the bacteria, in some cases with the fungi (among the Hyphomycetes, under *Oospora*,<sup>7</sup> or as a Mucedineous group with tendencies towards an erect Isarioid habit<sup>8</sup>) and in some cases as a separate group forming the connecting

<sup>2</sup> Merrill, E. D. and Wade, H. W. *Phillip. Jour. Sci.* 14: 55-69. 1919.

<sup>3</sup> Mello, F. de, and Fernandes, J. F. S. A. *Mem. Asiatic Soc. Bengal*, 7: 103-138. 1919.

<sup>4</sup> Harz, C. O. *Jahresber. Tierarzneischule, München*, 1877-78, 125-140.

<sup>5</sup> Krainsky, A. *Centrbl. Bakt.* II, 41: 649-688. 1914; Waksman, S. A. *Soil Sci.*, 8: 71-215. 1919; Conn, H. J. *N. Y. Agr. Exp. Sta. Tech. Bul.* 60, 1917; Drechsler, C. *Bot. Gaz.*, 67: 65-83, 147-168. 1919; Lieske, R. *Morphologie und Biologie der Strahlenpilze.* Leipzig. 1921; Orskov, 1923 (p. 275).

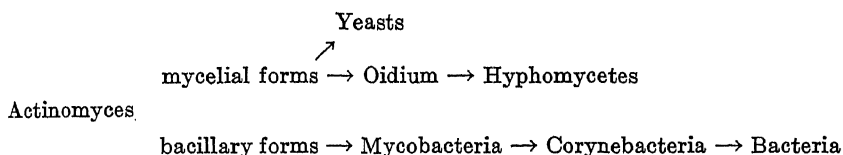
<sup>6</sup> Lehmann and Neumann, 1921 (p. XI); Lachner-Sandoval, V. *Über Strahlenpilze.* Strassburg, Diss. 1898; Breed, R. S. and Conn, H. J. *Jour. Bact.*, 4: 585-602. 1919; 5: 489-490. 1920; Buchanan, 1925 (p. XI).

<sup>7</sup> Savageau and Radais. *Ann. Inst. Past.*, 6: 242-273. 1892.

<sup>8</sup> Drechsler, see <sup>5</sup>.

link between the bacteria and the fungi.<sup>9</sup> The actinomyces may look like true bacteria in ordinary stained preparations, due to the fact that the mycelium is very fine and is readily broken up into various fragments, and the conidia look very much like bacterial cells. In unstained preparations, however, such as are used for the direct examination of the colony, or when stained by some special method,<sup>10</sup> the branching and manner of spore formation can be seen to resemble that of a true fungus.

Physiologically, the actinomyces are differentiated from both fungi and bacteria. The fungi are characterized, as a rule, by having greater resistance to acids than the bacteria. The actinomyces, with some few exceptions, are even more sensitive to acids than the majority of the bacteria developing on the common plate. A large number of bacteria are characterized by the formation of various gases from carbohydrates; the actinomyces are not known to form any gas from carbohydrates except CO<sub>2</sub>, which is a product of the energy metabolism of most living forms; in this respect they behave like the fungi. The apparently close relationships which the actinomyces have with both bacteria and fungi led to the assumption that the actinomyces should be looked upon as the ancestral form of both bacteria and fungi, according to the following scheme:



The actinomyces are thus found to partake of the nature of both bacteria and fungi and are to be classed tentatively as intermediate forms. But they cannot be classified unreservedly with the fungi, particularly with the Hyphomycetes which is a rather loose conglomerate of various forms. The property of acid-fastness correlated with a certain type of pathogenicity (formation of tubercles) and with cross immunity reactions with the acid fast bacteria, points to a certain relation, of at least some pathogenic forms, to the bacteria.

*Species differentiation.* Large numbers of actinomyces have been described by various investigators. The greater the number of forms

<sup>9</sup> Lieske, 1921, p. 276; Lehmann and Neumann, 1921, p. XI. Claypole, E. Jour. Exp. Med., 17: 99-116. 1913.

<sup>10</sup> Waksman, S. A. and Curtis, R. E. Soil Sci., 1: 99-134. 1916; Waksman, 1919 (p. 276); Drechsler, 1919 (p. 276).

collected, the more difficult is the division into species. The variability of these organisms is such that out of a dozen isolations from a plate not more than two or three may be alike. Even forms recognized to be alike on one medium will be found to be different when grown on another medium. Sometimes two forms found to be alike on several media, will be found to show distinctive characters on further study.<sup>11</sup> This is especially true of the cultural and biochemical characters and to some extent even of the morphology of the organisms.

It is important first to obtain absolutely pure cultures of the actinomyces. Even then, an attempt to designate a species by the sum total of its morphological and physiological characters may not give very satisfactory results. It is best to separate the actinomyces not into individual strains, but into groups, defined by a sum total of certain definite morphological and physiological characters. The amount of variability within each group and the amount of overlapping between two groups is something that cannot be definitely established and must be left to the judgment of the student.

The species differ primarily in the length of vegetative mycelium, type of aerial mycelium, absence or presence of spores, method of spore formation, shape and color of colony, formation of soluble pigment, oxygen requirement, production of diastatic and proteolytic enzymes and a number of other morphological and physiological characters. These vary in quantity as well as in quality, not only under the influence of environmental conditions but even on continued cultivation under the same conditions. The soluble pigment may be lost or changed in kind, and even the color of the aerial mycelium may be modified as well as the very property of forming aerial mycelium may be lost.

An important advance in the study of this group has been the introduction of synthetic media. The variability of the organisms mentioned by Lieske was due largely to the use of non-synthetic nutrient agar, which is variable in composition. Two media made up exactly alike, but differing merely in the amount of boiling, period of sterilization, a slight change in ratio between the carbon and nitrogen sources, or in reaction and concentration of nutrients, will show distinctive differences for the various organisms. By growing these on synthetic media, with due allowance to a certain amount of variability, certain definite characters may be established.

*Methods of study.* For determining the abundance of actinomyces in the soil, the general media used for the determination of numbers of

<sup>11</sup> Conn, H. J. N. Y. Agr. Exp. Sta. Tech. Bul. 83, 1921.

bacteria can be employed (p. 15). For the study of cultural and morphological characters, several media have been suggested:

1. Glucose agar:
  - 10 grams glucose
  - 0.5 gram  $K_2HPO_4$
  - 0.5 gram asparagine
  - 15 grams agar
  - 1000 cc. distilled water
2. Malate-glycerol agar:
  - 10 grams calcium malate
  - 0.5 gram  $NH_4Cl$
  - 0.5 gram  $K_2HPO_4$
  - 10 grams glycerol
  - 15 grams agar
  - 1000 cc. distilled water
  - Reaction adjusted by means of NaOH to pH = 7.0
3. Citrate-glycerol agar
  - Same as 2, except that calcium citrate is used in place of malate
4. Czapek's agar (p. 226)
5. Starch agar:
  - 10 grams starch is suspended in 800 cc. of water and boiled down to 500 cc. 500 cc. water to which are added 1 gram  $K_2HPO_4$ , 1 gram  $MgSO_4$ , 1 gram NaCl, 2 grams  $(NH_4)_2SO_4$ , 3 grams  $CaCO_3$ , 10 grams agar. The two solutions are mixed and tubed.
6. Gelatin:
  - 15 per cent Gold Label gelatin in distilled water.

The first four media are used for the study of general cultural characters of the organisms and are well suited for morphological studies; the starch and gelatin media supply information on two of the most important physiological properties of the organisms, namely the diastatic and proteolytic.

For microscopic examination, one of the two following methods may be used:

1. Method of Henrici: Melted and cooled agar is inoculated with the specific organism and spread in a thin film on flamed slides, which are then incubated in a sterile moist chamber. After growth has taken place, the slides are dried, fixed in alcohol and stained. The entire colony, with both vegetative and aerial mycelium, can thus be examined in an undisturbed condition.

2. Method of Drechsler: The organism is grown on a synthetic medium and, when the culture is fully developed, the whole colony is cut from the agar and removed, as carefully as possible, from the tube or plate. A slide smeared with albumin fixative is now brought into firm contact with the surface mycelium and then separated from it, precautions being taken to avoid any sliding of the two surfaces on each other. If the growth is not too young, the upper portions of the aerial mycelium will be left adhering to the slide without any series disarrangement; killing and fixation may be effected at once. The material is then stained and mounted in balsam. Preparations, in which the spore chains have commenced to disintegrate, are impaired by the large masses of free spores. The most convenient fixative agent is 95 per cent alcohol. As a stain, Haidenhain's iron-alum haemotoxylin is good for protoplasmic structures. Delafield's haemotoxylin, allowed to act for 24 hours with the proper degree of decolorization, yields deeply stained, clear preparations showing distinctly the vacuoles, metachromatic and nuclear structures, and septa.



*Nature of growth on artificial media.* The term colony is used incorrectly in designating a mass of growth of an actinomyces, since it is merely a mass of mycelium developing out of a single spore, and not a colony in the sense of bacterial growth. Each spore or piece of mycelium separated from the colony is capable of individual existence, developing into a new colony. The single-celled colony of an actinomyces is characteristic and is easily distinguished from that of bacteria or fungi. It is usually round and develops in the form of a semi-circle into the medium (No. 120, Pl. XIII). The colonies are mostly compact, leathery, adhering to the medium, the surface being either flat or elevated; the outer zone is smooth, round as seen with the naked eye, and has a fringe of minute hyphae projecting for a short distance into the medium when observed under the low power. The surface is usually dry and often presents a conical appearance; it is either free from any aerial mycelium, or covered with a chalky (mealy, mildewy) white, drab or grey aerial mycelium, or with an abundant cottony, fuzzy, white, red or grey aerial mycelium.

The subsurface growth of different organisms is little differentiated from one another, being usually of white-greyish or yellowish color; but the surface growth and the subsurface growth which may develop up to the surface, have a characteristic appearance on synthetic media. The growth of some organisms presents a smooth surface, while others have a much folded, or lichnoid surface; still others form a fine network on the surface. These characters are not constant but change with the composition of the medium and age of culture even on artificial culture media.

Another characteristic of the growth of some species is the formation of "fairy rings" consisting of concentric spore-bearing rings and spore-free rings disposed in zones; the zones are also formed in the spore-free colony. It has been suggested<sup>12</sup> that ring formation by fungi is a result of diffusion of injurious substances present or formed in the medium or due to the action of light, which produces a change in transpiration and temperature.

*Vegetative mycelium.* The vegetative growth consists of a mycelium composed of profusely branching hyphae (Nos. 121-122, Pl. XIII), the terminal growing portions of which are densely filled with protoplasm. Vacuoles are formed in the mycelium; these vacuoles increase in size towards the center of the thallus. They are possibly associated with

<sup>12</sup> Munk, M. Centrbl. Bakt. II, **32**: 353-375. 1912. Biol. Centrbl., **34**: 621. 1914.

the presence of metachromatic granules which are often mistaken for micrococci or bacterial endospores. The actinomyces may be divided into two groups on the basis of the length of the hyphae: (1) those forming long, abundantly branching hyphae and (2) those which show on the slide only short unbranching pieces of mycelium or rods. According to Lieske, the aerobic forms, both spore-forming and non-spore forming, growing fast into the substratum, belong to the first group, while the anaerobic pathogenic forms are included in the second. The composition of the medium greatly influences the length of the hyphae so that, with 20 per cent of cane sugar in the medium or in strongly alkaline media, a long mycelial species was found to form a very short mycelium.

According to Drechsler, the vegetative mycelium is infrequently and irregularly septated. While in some forms transverse walls appear with somewhat greater frequency, there are none in which septation approaches any pronounced degree of regularity or closeness. The branches are formed by the elongation of lateral buds arising some distance back from the growing point of an axial filament. The branch thus produced gives rise to secondary branches by lateral proliferation. In addition to the typical monopodial branching, true dichotomy may occasionally occur.<sup>13</sup> In old cultures, certain swellings of the terminal ends of the hyphae are observed; these may also be formed under abnormal growth conditions, as in concentrated media or in the presence of substances like caffeine. These swellings are somewhat similar to the clubs formed by pathogenic actinomyces in the animal body. The separation of actinomyces on the basis of these formations, or the so-called involution forms, is open to criticism. The acid-fast staining reaction of pathogenic actinomyces seems to be a constant property as long as the organisms are growing in the tissues of the infected animals. However, it cannot be used for the differentiation of soil forms.

*Spore bearing mycelium.* Most actinomyces produce an aerial mycelium on suitable substrates either in the form of a mat of fructifications or numerous erect sporodochia (coremia). In any case each individual fructification represents a well characterized sporogenous apparatus, consisting of a sterile axial filament bearing branches in an open racemose or dense capitate arrangement. The primary branches may function directly as sporogenous hyphae, or may produce branches of the second and higher orders. In the latter case sporogenesis is confined to the terminal elements and the hyphal portions below the points of attachment of branches remain sterile.

<sup>13</sup> Macé, E. Compt. Rend. Acad. Sci., 106: 1622. 1888; Neukirch, H. Über Strahlenpilze. II, 1902.

The morphology of the spore-bearing hyphae of the various actinomyces exhibits distinct individuality and can readily serve as a basis for specific differentiation. The specialized, sporogenous hyphae are distinguished from the sterile hyphae of the aerial mycelium at an early stage of their development. While the diameter of the sterile mycelium which arises through the elongation of the growing filament tip shows very little subsequent increase in thickness, the sporogenous hyphae are in the beginning thinner than the axial hyphae from which they are derived. Increase in thickness of the sporogenous hyphae follows after the final linear extension has been attained. The final diameter of the sporogeneous hyphae may be less or appreciably more than that of the vegetative hyphae.

Orskov suggested the division of actinomyces into three genera:

1. Sporogenic fungi. The spores germinate and develop into a unicellular substrate or vegetative mycelium that does not divide spontaneously. Out of this mycelium arises an aerial mycelium of hyphae that are thicker than those of the vegetative mycelium. The aerial mycelium later divides by the breaking up of the protoplasm into regularly sized parts. These are separated from one another by constriction of the thread membrane between the individual elements. These organisms grow in the form of flakes in liquid media, usually at the bottom. The name *Cohnistrepthrix* was retained for this genus.

2. An initially undivided substrate mycelium is formed with an early development of aerial hyphae, which are of the same thickness as the vegetative hyphae. Both substrate and aerial mycelium divides spontaneously into segments of irregular size and shape, by the formation of transverse walls. The organisms belonging to this group produce an early surface growth on liquid media. It was suggested to reserve the name *Actinomyces* for this genus.

3. A unicellular, delicate, branching mycelium, at the extreme tips of which single oval spores are formed. The organisms produce growth in liquid media, in the form of small firm grains at the bottom and along the glass. The name *Micromonospora* was retained for this genus.

All the three groups are found abundantly in the soil, especially the second one, to which most of the soil actinomyces so far studied belong. Jensen<sup>14</sup> isolated from Australian soils ten strains of organisms belonging to the *Micromonospora* group and found that they may occupy 5-8 per cent of the total colonies of Actinomyces developing on the plate. Their abundance is increased by the addition of cellulose or lignin to the soil.

A classification found convenient for the study of soil actinomyces

<sup>14</sup> Jensen, H. L. Proc. Linn. Soc. N. S. Wales, 55: 231-248. 1930.

is tentatively suggested here. It is based on the formation of sporogenous hyphae.

1. Presence of substrate mycelium alone and no aerial mycelium formed on synthetic or organic media.

2. The aerial mycelium consisting of very long filaments, rarely branching and mostly sporogenous almost to the point of origin in the nutritive mycelium, without any coiling. This group can be subdivided into two sub-groups: (a) long mycelium and little branching (No. 123, Pl. XIII); (b) short mycelium and abundant branching (No. 124, Pl. XIII).

3. Maturation of the sporogenous hyphae associated with the formation of characteristic spirals. A flexuous habit of the young filament early manifesting the tendency towards the coiling condition which becomes more definite with continued elongation. This group can be also divided into sub-groups on the basis of the obliquity of the spiral, diameter of turns, construction with reference to the dextrorse and sinistrorse condition (constant characters, according to Drechsler). The spirals range from open (No. 127, Pl. XIII), barely perceptible turns to strongly compressed spirals (No. 126), so that the adjacent turns are in continuous contact. The number of turns ranges from two or three to twenty or more.

4. Sporogenous hyphae formed in knot-like groups of three or four along a central hypha (No. 125, Pl. XIII).

Group 3 is most abundant in the soil and is, therefore, described in most detail. The representatives of this group form sporogenous hyphae ranging from the straight mycelium with a barely perceptible waviness of group 2 to the strongly compressed spirals, resembling a closed fist. The diameter of the spirals is usually somewhat in an inverse ratio to the number of turns characteristic of the species. It must be noted here, however, that the nature and composition of the medium greatly influence the morphology of the organisms: Certain species belonging to group 4 (*Act. reticuli*) when grown on Czapek's agar, can be classified with group 3 when grown on nutrient agar or even on certain inorganic media.

By comparing the relation of the sporogenous branches to each other and the axial filaments, Drechsler noted two main types approaching each other in apparently intermediate forms, but quite distinct at the extremes: (a) erect dendroidic type in which the sequence of development of the sporogenous hyphae is successive; fructification starts from a single erect hypha with a spiral termination; sporogenesis commences at the tip by the insertion of regularly spaced septa, and proceeds downward toward the base of the filament; (b) prostrate, racemose type in which development of fructifications is more nearly simultaneous. In most cultures, however, both types are combined.

The various species are usually characterized by clearly defined septation and have been separated by Drechsler into three different groups, on the basis of the disposition of their septa and development of their spores.

In the first group, the cross walls in the sporogenous hyphae remain without any very pronounced change and continue to separate the adjacent cells until they have developed into a chain of mature contiguous spores. The insertion of these septa progresses from the tip toward the base and does not break the physiological continuity of the hyphae. Food material is, apparently, readily transported through these septa to the young spores at the termination, since the spores increase in size and may deposit a wall of measurable thickness.

In the second group the septa apparently split into halves, which are then drawn apart by the longitudinal contraction of the individual protoplasts.

In the third group the cross-walls first undergo a deep constriction which, by involving the ends of the young cylindrical spores, gives to the latter an elongated ellipsoidal shape. The constricted septum now gradually loses its staining properties, and appears to become slightly drawn out in a longitudinal direction. A preparation stained with Delafield's haematoxylin usually shows many old spore chains in which the individual spores are thus connected by hyaline isthmuses. Occasionally an isthmus may be found with a remnant of the old deep staining septum still unchanged in its center.

Beyond these three types of sporulation another must at least be provisionally recognized, in which septa are either absent from the developing sporogenous hyphae or are not demonstrated by the use of ordinary stains. The protoplast appears to contract at regular intervals, yielding a series of non-contiguous spores, held together for a time by the connecting segments of evacuated filament wall.

The germination of the spores takes place in dilute nutrient solutions. The spore first swells, then one to four germ tubes are produced. The number of germ tubes is more or less characteristic of the species.

*Utilization of carbon compounds by actinomyces as sources of energy.* Various species of actinomyces utilize a number of sugars and higher alcohols as sources of energy, with inorganic sources of nitrogen, especially glucose, starch, maltose and glycerol. Lactose, sucrose and inulin are utilized to a less extent, depending upon the ability of the organism to form the corresponding enzyme. Arabinose, mannitol and cellulose<sup>15</sup> serve as good sources of carbon only for certain species. Various organic acids, such as succinic, malic, tartaric, and citric, are well utilized, but formic, acetic, propionic, butyric, valerianic, lactic, benzoic and oxalic

<sup>15</sup> Krainsky, A., 1914 (p. 276); Zhur. Opit. Agron., 14: 255-261. 1913; Waksman, S. A. Jour. Bact., 4: 307-330. 1919.

are not readily available.<sup>16</sup> Proteins and amino acids are excellent sources of carbon.<sup>17</sup> Lieske<sup>18</sup> employed a solution of 1 per cent urea, traces of  $K_2HPO_4$  and  $MgSO_4$  and 2 per cent of the corresponding carbon source in distilled water. He found that the aerobic, saprophytic strains utilized maltose, lactose, levulose, dextrin, glucose, glycerol, glycogen, inulin, mannitol, asparagine, human blood serum, and sucrose with decreasing efficiency in the order named. Only one of the two strains decomposed starch and none attacked cellulose. Failure to obtain growth with cellulose is more often due to the fact that the proper method has not been employed. The same is true, to a less extent, of the utilization of starch which is commonly found to be one of the best sources of energy for the majority of actinomyces (except a few human pathogenes). Some strains utilize ethyl and methyl alcohol and, to a small extent, tannin. Amygdalin, caffeine, sodium soap and potassium acetate may also be assimilated.<sup>18</sup>

Actinomyces can grow on butter fat; growth is accompanied by a definite increase in acidity.<sup>19</sup> By the use of a modification of Eijkman's method, it was demonstrated that a large number of actinomyces are able to produce fat splitting enzymes.<sup>18</sup> This was accomplished by adding 1-3 per cent of the fat to the molten agar, shaking well, pouring plates, then inoculating. On adding an indicator, such as litmus or brom phenol blue, no acidity could be indicated. The fatty acids are neutralized, as can be readily demonstrated by the formation of crystals on the plate. Some actinomyces can utilize rubber as a source of carbon.<sup>20</sup> Lantzsch<sup>21</sup> isolated an Actinomyces growing in distilled water containing quartz or in a medium very poor in organic matter; this organism was found to be identical with *Bac. oligocarophilus* of Beijerinck and it could assimilate CO as well as the higher aliphatic hydrocarbons, except benzol and xylol.

*Nitrogen utilization.* Proteins form good sources of nitrogen; the same is true of peptones and various amino acids. Gelatin (15 per cent in distilled water, neutralized or reaction unadjusted) is liquefied in 3 to 5 days, at 18° to 20°C., but some species produce only a very narrow liquefied zone after 30 to 40 days. Some do not produce any

<sup>16</sup> Salzmann, P. Diss. Königsberg. 1901.

<sup>17</sup> Münter, F. Centrbl. Bakt. II, 36: 365-381. 1913.

<sup>18</sup> Lieske, 1921 (p. 276).

<sup>19</sup> Jensen, O. Centrbl. Bakt. II, 8: 171. 1902.

<sup>20</sup> Shnngen, N. L. and Fol, J. G. Centrbl. Bakt. II, 40: 87-98. 1914.

<sup>21</sup> Lantzsch, K. Centrbl. Bakt. II, 57: 309-319. 1922.

pigment, others form a brown to purple-brown soluble pigment on gelatin. A number of species hemolyze red blood cells and produce a clear zone around the colony when grown on blood agar. On the basis of milk coagulation the actinomyces can be divided into several groups.<sup>21a</sup>

1. Rapid coagulation of milk, followed by rapid liquefaction of the coagulum, so that the tube of milk becomes clear in 6 to 7 days.
2. Rapid coagulation of the milk followed by slow liquefaction.
3. Slow coagulation followed by a rapid liquefaction.
4. Digestion of the milk proteins without any previous coagulation. This tends to indicate that the rennet-like and proteolytic enzymes are distinct.

Among the inorganic compounds, ammonium salts are utilized well, in the presence of sufficient buffering agents, especially silicates.<sup>22</sup> Nitrates are reduced first to nitrites before they are assimilated. Nitrites used in very low concentrations (0.01 per cent) are also readily assimilated. Creatinine can be used as a source of nitrogen. Urea and acetamide are assimilated only to a limited extent by the majority of organisms, while a few forms may utilize the substances readily.<sup>23</sup> Lieske found that urea forms an excellent source of nitrogen; asparagine was only scantily available; uric and hippuric acids were not assimilated at all.

The proteins are decomposed, with the formation of ammonia, even in the presence of available carbohydrates, such as glucose. Guittonneau<sup>24</sup> demonstrated that certain actinomyces are capable of producing urea from proteins, both in the presence and absence of glucose, in addition to ammonia.

*Oxygen requirement.* The influence of oxygen tension on actinomyces is still an undecided question. There is no doubt that the majority of soil actinomyces are aerobic. The fact that some are able to grow deep into the medium would indicate ability to grow under semi-anaerobic conditions. Beijerinck<sup>25</sup> classified the actinomyces as facultative anaerobes. Lieske, among others, found that the actinomyces acting as animal pathogens are anaerobic, while the saprophytic forms are chiefly aerobes. *A. scabies* does not germinate in the absence of oxygen; the amount of available oxygen is a limiting factor both for germination and

<sup>21a</sup> Waksman, S. A. Jour. Bact., 4: 189-216. 1919.

<sup>22</sup> Münter, F. Centrbl. Bakt. II, 39: 561-583. 1914.

<sup>23</sup> Waksman, S. A. Jour. Bact., 5: 1-30. 1920.

<sup>24</sup> Guittonneau, G. Compt. Rend. Acad. Sci., 178: 1383-1385. 1924.

<sup>25</sup> Beijerinck, M. W. Centrbl. Bakt. II, 6: 2-12. 1900.

growth.<sup>26</sup> Other investigators<sup>27</sup> pointed out that the recognition of strict aerobes or anaerobes is based upon errors of technic, since in no instance could a strict aerobe or anaerobe be obtained.

*Influence of temperature, drying and radiation.* Some actinomycetes grow slowly at temperatures of 3° to 6°C. Good growth of most species is obtained at 6° to 38°, 13° to 32°C. being the optimum for the majority of soil forms. Few organisms can thrive at 40–42° and none will grow at temperatures above 46°C. except the thermophilic forms. The temperature limits may be raised a few degrees by gradual adaptation.

Low temperatures (even 8° to 10°C.) do not injure the majority of actinomycetes. High temperatures are very injurious. Some species are killed at 45°.<sup>28</sup> The thermal death point of most actinomycetes is 75°. The mycelium of some organisms may survive at 60°, but is killed in 20 minutes at 70°; the spores may survive 75° for 20 minutes but are killed in 30 minutes. Some soil organisms are able to withstand even somewhat higher temperatures. The actinomycetes spores resemble the spores of true fungi in that they do not possess the degree of resistance of the bacterial spores, and are readily destroyed at temperatures a few degrees (about 5°C) above the destructive temperature of the mycelium.

The existence of actinomycetes in the soil which can grow readily at 60°C has been established.<sup>29</sup> These forms are especially abundant in the upper layers of forest and garden soils; they are also found abundantly in dry peat, grass, hay and straw. The thermophilic forms do not represent one species, but several distinctly different forms. Various investigators<sup>30</sup> suggested that in the self-heating of straw and other plant materials, the thermophilic actinomycetes have a chance to develop and that the addition of straw or manure to the soil helps to spread the organisms. The thermophilic species multiply rapidly in the soil when the soil is warmed during the hot summer months by the direct rays of the sun. The fact that they are found in cold places where the minimum temperature (40°) for growth of thermophilic forms is never reached, such as forest soils, deep subsoils, and frozen soil, led Lieske to con-

<sup>26</sup> Sanford, G. B. *Phytopath.*, **16**: 525–548. 1926.

<sup>27</sup> Musgrave, W. E., Clegg, M. T. and Polk, M. *Philip. Jour. Sci., B., Med. Sci.*, **3**: 447–544. 1908.

<sup>28</sup> Foulerton, A. G. R. and Jones, C. B. *Trans. Path. Soc. London*, **53**: 56–127. 1902.

<sup>29</sup> Globig. *Ztschr. Hyg.*, **3**: 294. 1888; Gilbert. *Ztschr. Hyg.*, **47**: 383–406. 1904.

<sup>30</sup> Mische, H. *Die Selbsterhitzung des Heus*. G. Fischer. Jena. 1907; Noack, K. *Jahrb. wiss. Bot.*, **51**: 593–648. 1912.



clude that some of the common soil actinomyces give rise to mutants which develop at the higher temperatures. This whole subject deserves further careful study.

All actinomyces are very resistant to drying, as indicated by the fact that they are found abundantly on dry straw, hay and soil. Berestneff<sup>31</sup> inoculated some ears of grain with a pure culture of *A. violaceus* and found that he could obtain a pure culture of the organism from the dry material after preserving it for ten years. Lieske found that only the spores and mycelium of saprophytic organisms were viable after they had been preserved on filter paper in a desiccator for eighteen months. This points to the fact that pathogenic actinomyces are not carried over for any long time in dry soil or straw, as is often assumed.

Direct sunlight does not exert any injurious effect upon the actinomyces and does not modify their growth. Exposure to ultraviolet rays for 10 minutes does not cause any injury; after one hour, the organism is definitely affected but not destroyed.

*Influence of reaction and salt concentration.* It has been generally observed that alkalis and alkali substances favor the development of actinomyces, while acids and acid substances injure their activities. This is particularly true of inorganic acids, since the organic acids are utilized to some extent by the organisms and are thus broken down. The limiting acid reaction for the majority of soil actinomyces is pH 4.8 to 5.0, although some species may grow at as high an acidity as pH 3.0 to 4.0. The optimum is pH 7.0 to 8.5. On the alkaline side, the majority of organisms will still grow at pH 8.6 to 9.0, while some will grow even at more alkaline reactions. This fact can be utilized in adjusting the reaction of the soil so as to prevent the development of *A. scabies* causing potato scab.

Gillespie<sup>32</sup> was the first to point out the fact that soils having a reaction of pH 4.8 or less are free from scab, while those having a reaction more alkaline than pH 4.8 are apt to have scab. Various strains of *A. scabies* may behave somewhat differently: some may be inhibited in their development at pH 5.0, while others only at pH 4.6. The organisms are also able to withstand a somewhat greater acidity in the soil than in solution.<sup>33</sup> The reaction of the medium is usually changed by the growing organism to less acid or more alkaline;<sup>34</sup> this is true of media contain-

<sup>31</sup> Berestneff, N. M. Centrbl. Bakt. I, Ref., 40: 298. 1907.

<sup>32</sup> Gillespie, L. J. Phytopath., 8: 257-269. 1918.

<sup>33</sup> Waksman, S. A. Soil Sci., 14: 61-79. 1922.

<sup>34</sup> Näslund, C. and Dernby, K. G. Biochem. Ztschr., 138: 477. 1923.

ing proteins, amino acids and  $\text{NaNO}_3$ . The proteins and amino acids are decomposed with abundant formation of ammonia. With ammonium salts, the ammonium radical is rapidly used up, thus allowing the medium to become acid. No acids are formed from carbohydrates.

Highly acid soils seem to harbor more acid tolerant organisms. Jensen<sup>35</sup> isolated from acid peat soils of pH 3.4 to 4.1 four strains of actinomycetes (*A. acidophilus*), which are not only capable of living on acid media but produce their best growth at pH 4.0 to 5.0.

Certain actinomycetes will grow in media containing 12 per cent glycerol or 7 per cent  $\text{NaCl}$ .<sup>36</sup> In the presence of 18 per cent glycerol and 9 per cent  $\text{NaCl}$ , no growth was observed. Actinomycetes were found<sup>37</sup> to grow in the presence of 5 per cent of  $\text{KCl}$ ,  $\text{NaCl}$ ,  $\text{KNO}_3$ ,  $\text{NaNO}_3$ , as well as mixtures of these, but spore formation was depressed. Ten per cent of the salts repressed the growth of all forms except one. Magnesium salts proved much more injurious. Small quantities of alkali earths stimulated, while larger quantities injured growth and spore formation. Difficultly soluble carbonates had little effect.  $\text{AgNO}_3$  inhibited growth completely; 0.1 per cent  $\text{Cu}$  as  $\text{CuSO}_4$  or  $\text{CuCl}_2$  was injurious,  $\text{HgCl}_2$  was less injurious. Lead nitrate and iron salts were least injurious. Lieske could not observe any diminution in growth of various actinomycetes when 15 per cent cane sugar was added to nutrient bouillon. Slight growth was obtained in the presence of 20 per cent cane sugar; no further growth was observed in the presence of 30 per cent sugar.

*Influence of organic poisons.* The addition of benzol to the soil was found to stimulate the development of actinomycetes but they are injured by the application of carbon bisulfide.<sup>38</sup> These organisms are not very sensitive towards chemical poisons but cannot resist the action of metallic salts. The limit of the action of a poison can be changed by gradual adaptation. Of the dyes, methylene blue, methyl violet and gentian violet are most toxic, preventing growth in 1:500,000 dilution in nutrient bouillon.

*Reduction of nitrates and other compounds.* The majority of actinomycetes species are able to reduce nitrates to nitrites, but not to ammonia nor to atmospheric nitrogen.<sup>39</sup> By using the ordinary Czapek's solution,

<sup>35</sup> Jensen, H. L. Soil Sci. 25: 225-236. 1928.

<sup>36</sup> Neukirch, 1902 (p. 281).

<sup>37</sup> Münter, F. Centrbl. Bakt. II, 44: 673-695. 1916.

<sup>38</sup> Störmer, K. Centrbl. Bakt. II, 20: 282-286. 1908.

<sup>39</sup> Fousek, A. Mitt. landw. Lehrkanz. K. K. Hochsch. Bodenk. Vienna, 1: 217-244. 1912.

nitrite formation can be demonstrated to run parallel to the growth of the organism. Some starch solution and 0.5 per cent  $\text{KNO}_3$  may be added to ordinary nutrient agar, which is then placed in Petri dishes, cooled and inoculated with the organism in question. After the organism has developed for a few days, the plates are covered with a dilute solution of potassium iodide, to which some hydrochloric acid is added. The plates, in which the nitrate is reduced to nitrite, are colored blue since the nitrite liberates the iodine from the potassium iodide.<sup>18</sup> This method, however, is not so reliable as the common method ( $\alpha$  naphthalamine + sulfanilic acid) of nitrite estimation, since the starch may be decomposed by the diastatic enzymes.

Selenium and tellurium salts (0.01 per cent concentration) are reduced by numerous actinomyces to elementary selenium and tellurium; the colonies are colored deep red and deep black respectively, due to the fact that the metals are deposited within the mycelium. The phenomenon is intracellular. According to Husz,<sup>40</sup> various actinomyces isolated from the soil can reduce organic arsenic compounds, similar to *Pen. brevicaulis*, with the production of the characteristic garlic odor. These results could not be confirmed by Lieske. This may be due either to the difference in the organisms employed or difference in methods.

*Production of odor.* Most actinomyces grown on organic or synthetic media produce a characteristic odor of freshly plowed soil. This is particularly true of the organisms possessing a mildewy aerial mycelium. The odor varies from earthy to musty. It was thought at first<sup>41</sup> that this odor was formed by a specific organism. It was soon found that this is the property of a large number of species and the odor production is in itself a variable factor. The non-spore-forming organisms usually do not produce any odor. The intensity of the odor depends on the composition of the medium. Carbohydrates, particularly glycerol, stimulate the odor formation. The odoriferous substance has not been isolated yet. A few actinomyces, particularly thermophilic forms, produce a pleasant fruity odor.

*Pigment formation.* The property of pigment formation is widespread among the actinomyces. No culture can be considered non-chromogenic until it has been studied on protein media and a variety of protein-free media. Three different kinds of pigment should be considered: that of the vegetative mycelium, of the aerial mycelium, and pigment dissolved out into the medium. The aerial mycelium is usually

<sup>40</sup> Husz, H. Ztschr. Hyg., 76: 361. 1914.

<sup>41</sup> Rullmann. Diss. München. 1895.

colored white, gray or buff, sometimes red, yellow, brown, light green or bluish-green. The vegetative or substrate growth is usually colored gray, red, yellow, orange, brown or black. Among the soluble pigments, purple, brown, black and yellow are predominant; red, blue and green are also formed by some species. Soluble brown to purple pigments are very common on protein media. A slight difference in the composition of the medium has an important influence upon the pigment formation by actinomyces.

Various attempts have been made to explain the nature of the dark brown pigments produced on organic media. Beijerinck<sup>42</sup> suggested that the brown pigment is a result of quinone-formation, as shown by the fact that ferri-salts color the brown-colored gelatin black and the gelatin itself is made insoluble due to the action of the pigment. In the presence of HCl, iodine is liberated from potassium iodide. The formation of the pigment has also been ascribed<sup>43</sup> to the action of an enzyme, as in the case of tyrosine media coloring black. But the organisms must be able to synthesize their own tyrosine and produce a brown or dark pigment since the pigment is produced also on tyrosine-free media. Some actinomyces form blue or green pigments, particularly when freshly isolated. The rapidity of gelatin liquefaction and pigment formation are utilized for diagnostic purposes. The characterization of an organism by the nature of the pigments formed has sometimes led to duplications, as in the naming of one organism, on the basis of formation of a red and blue pigment, *A. violaceus ruber* and *A. tricolor*.<sup>44</sup> The pigment acts as a natural indicator. On media which are slightly acid (pH 6.0), the pigment is at first red, then, with a change of reaction of the medium to alkaline, the pigment becomes blue.

*Variability.* The actinomyces show, in their morphological and physiological characteristics, greater variability than any other group of organisms. Size, shape and color of colonies, abundance and length of mycelium, spore formation are determined largely by the composition of the medium and age of culture. When organisms are named merely on the basis of pigment production on complex media (*A. chromogenus*, *A. sulfureus*, etc.), or on the basis of color of aerial mycelium (*A. albus*, *A. niger*), on the basis of ring formation in aerial mycelium (*A. annulatus*), we are merely utilizing variable properties of the organisms as

<sup>42</sup> Beijerinck, 1900 (p. 286).

<sup>43</sup> Sano, K. Diss. Würzburg. 102.

<sup>44</sup> Waksman and Curtis, 1916 (p. 277); Wollenweber, H. W. Arb. Forsschung. Kartoffel. H. 2, Berlin. 1920.

some distinguishing characters. When the cultures are transferred to other media, or even when cultivated continuously on the same medium, the pigment may change and rings may no longer be formed. The species thus lose their distinguishing characteristics and may become recognized as new species. In addition to using a group of morphological and physiological characteristics which are commonly employed for the species determination, one must also allow for the variability of the organisms. Observations should be made of the morphological and physiological characteristics on synthetic media for a large number of generations. Such characters as pigment production may change. The property of producing aerial mycelium may be lost and the character of growth changed. In some instances, cultures that lose the power of forming aerial mycelium regain it after cultivation in sterile soil.

Similar variability is found in the rapidity of gelatin liquefaction, action on milk, oxygen requirement, and odor production. One can readily observe in some actinomyces cultures the formation of sectors, differing in one respect or another from the rest of the growth. On transferring from such a sector to a fresh medium, an organism may be obtained which differs from the mother culture in some character such as color, presence of aerial mycelium, zone formation, pigment, etc. Lieske has thus isolated five new forms, in addition to the original, which were distinguished from one another by at least one character. Although the claim is put forth that pure spore cultures were used, no mention is made of the use of such a procedure as the Barber pipette, which would absolutely insure a single-spore culture.<sup>44a</sup>

*Species differentiation.* For a study of the cultural and biochemical characteristics of actinomyces, a group of media may be recommended, which will help to bring out the salient features.

1. Synthetic media, as described above:

- (a) Modified Czapek's agar
- (b) Krinsky's glucose agar
- (c) Malate-glycerol agar (Conn)
- (d) Citrate-glycerol agar (Conn)

Temperature of incubation 25°, period of incubation 7 to 15 days.

2. Gelatin, 15 per cent in distilled water, reaction unadjusted; temperature of incubation 16° to 18°C; period of incubation 30 days.

3. Sterile skimmed milk; temperature of incubation 25° and 37°; observations made daily.

4. Potato plug, 25°, for 7 days.

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<sup>44a</sup> See Jensen, H. L. Proc. Linn. Soc. N. S. Wales, 56: 79-98, 1931.

5. Starch agar, 25°, 10 to 15 days (test for diastatic strength).

6. Nutrient peptone agar, 25°, 7 to 15 days.

General morphology to be studied by direct examination of colony on plate by means of low power. For detailed study, the method of Drechsler may be used, magnification 1000 to 1200.

The cultural characteristics of actinomycetes in these media make possible the suggestion of the following tentative key for their differentiation, until a more permanent one based on morphological studies can take its place.

#### KEY TO THE IDENTIFICATION OF SPECIES OF SOIL ACTINOMYCES

(Based chiefly on physiological characteristics)

##### A. Formation of a soluble pigment on all media containing protein substances:

###### I. Pigment deep brown (chromogenus types):

###### 1. A brown pigment is produced on tyrosine agar:

- (a) Pigment dark brown; white to cream-colored growth on synthetic media; soluble brown pigment on synthetic media containing arabinose, glucose or lactose

*A. scabies*

A number of strains of Actinomycetes were isolated from potato scab lesions; it has been suggested that there is no justification for including all these organisms in one species.<sup>45</sup> It has also been shown that certain types of mangel-beet scab may be caused by *A. scabies*, while other types of scab (mound scab) are caused by another organism named *A. tumuli*.

- (b) Pigment faint brown; sulfur-yellow soluble pigment on creatinine solution; aerial mycelium on glucose agar is colored ochre to reddish ochre.....*A. olivochromogenus*

###### 2. Growth and aerial mycelium on synthetic agar green to dark-green; soluble brown pigment on synthetic media with most carbohydrates.....*A. viridochromogenus*

###### 3. Deep brown to black pigment on synthetic agar:

- (a) Weakly growing organisms; orange-red growth on potato plug; no visible aerial mycelium on synthetic agar

*A. purpeochromogenus*

- (b) Vigorously growing organisms; brown to black growth on potato plug; abundant cottony aerial mycelium on synthetic agar.....*A. pheochromogenus*

###### 4. Usually no action on milk (37°), accompanied by the darkening of the milk; mouse-gray aerial mycelium on synthetic agar; ammonium salts used readily with different sources of carbon

*A. aureus*

<sup>45</sup> Millard, W. A. and Burr, S. Ann. Appl. Biol., 13: 580-644. 1926; Millard, W. A. and Beeley, F. Ann. Appl. Biol. 14: 296-311. 1927.

## 5. Brown pigment never produced on synthetic media:

- (a) Aerial mycelium on synthetic media has lavender shade  
*A. lavendulae*
- (b) Aerial mycelium on synthetic agar is abundant, of a water green color.....*Actinomyces* 218
- (c) Whirl formation in aerial mycelium on synthetic agar:  
 (a') Growth colorless and aerial mycelium white  
*A. reticuli*  
 (b') Growth pink, aerial mycelium rose colored; nitrate reduction very abundant; fewer whirls  
*A. reticulus-ruber*
- (d) Growth on synthetic agar sulfur-yellow, with yellow aerial mycelium; barnacle-like, greenish-yellow growth on potato plug.....*A. flavus*
- (e) Growth on synthetic agar red colored, aerial mycelium abundant, orange colored; aerial hyphae usually do not form spirals.....*A. ruber*

## II. Soluble pigment on organic media faint brown, golden, yellow or blue:

- 1. Pigment blue, not always definite; soluble red pigment turning blue on synthetic agar.....*A. violaceus-ruber*
- 2. Pigment at first green on organic media and synthetic agar, property lost on continued cultivation, becoming brown on synthetic agar; aerial mycelium not produced on most media  
*A. verne*
- 3. Soluble pigment at first brown, property lost entirely on continued cultivation; growth and aerial mycelium on synthetic agar abundant, white.....*A. albus*
- 4. Soluble pigment yellowish green; growth on synthetic agar penetrating into the medium is pink.....*A. californicus*
- 5. Brown pigment produced only on certain protein media (usually gelatin and glucose broth, not nutrient agar):  
 (a) Growth on synthetic agar red to pink; no differentiated aerial mycelium or only scant white.....*A. bobili*  
 (b) Growth on synthetic agar colorless; aerial mycelium thin, rose-colored.....*A. roseus*  
 (c) Growth on carrot and potato rapidly spreading, enveloping the whole plug and destroying it rapidly, plug becoming colored deeply brown.....*A. griseolus*  
 (d) Red (vinaceous) soluble pigment on synthetic agar, often turning red-brown; white aerial mycelium  
*A. erythreus*

## B. No soluble pigment produced on gelatin or other protein media:

- I. Species strongly proteolytic; gelatin liquefied rapidly, milk clotted and peptonized rapidly.
- 1. Brown soluble pigment on synthetic agar; diastatic action very strong.....*A. diastaticus*
- 2. Rapid liquefaction of coagulated blood serum, strong hemolysis of blood (37°); aerial mycelium on synthetic agar has a tea-green tinge.....*A. griseus*

3. Yellowish green growth on starch plate with pinkish aerial mycelium; citron-yellow growth on synthetic agar. . . . . *A. citreus*
  4. Greenish-yellow growth on synthetic agar, gray powdery aerial mycelium, greenish-yellow soluble pigment. . . . . *A. flavovirens*
  5. Colorless growth on synthetic agar, white to grayish aerial mycelium, no spiral formation; thin reddish-brown growth on potato plug (purplish zone on plug); faint yellow pigment may develop on gelatin, blood and egg-media. . . . . *A. poolensis*
  6. Buff colored growth on glucose agar, violet-gray aerial mycelium; yellow growth on synthetic agar with light drab aerial mycelium; rapid destruction of potato plug. . . . . *A. olivaceus*
  7. Very scant colorless growth with scant white aerial mycelium on synthetic agar and on synthetic media containing  $\text{NaNO}_3$  as a source of nitrogen; abundant brown growth with white aerial mycelium and soluble brown pigment on glucose agar; growth on potato plug greenish turning black. . . . . *A. gelaticus*
- II. Proteolytic action weak:
1. Soluble pigment produced on synthetic agar:
    - (a) Pigment blue or blue-black. . . . . *A. violaceus-caesari*
    - (b) Pigment brown to almost black on all synthetic media with  $\text{NaNO}_3$  as a source of nitrogen. . . . . *A. exfoliatus*
  2. No soluble pigment on synthetic agar, although growth is colored:
    - (a) Growth turning black, diastatic action very strong:
      - (a') Growth on synthetic agar scant with abundant spirals in aerial mycelium, no invertase production  
*A. rutgersensis*
      - (b') No spirals on synthetic agar, characteristic green colored growth on protein-glycerol media  
*A. lipmanii*
      - (c') None or scant aerial mycelium on all media; growth abundant on synthetic agar (invertase positive); none or scant growth on blood agar and egg-media  
*A. halstedii*
    - (b) Growth orange colored on most synthetic and organic media; aerial mycelium pink. . . . . *A. fradii*
    - (c) Growth yellowish on synthetic and glucose agars; pinkish to cinnamon-colored on calcium malate agar; no growth on blood serum and egg media; none or only very scant and late aerial mycelium on most media. . . . . *A. alboflavus*
    - (d) Growth on synthetic media rose to red colored, aerial mycelium white, no visible action on milk. . . . . *A. albosporus*  
*Act. fulvissimus* described by Jensen produces no brown pigment on protein media but forms a typical golden pigment in all synthetic media.

Nearly one hundred species of actinomyces have been isolated from the soil and described. A much larger number, however, can readily be obtained. Some of them are of wide occurrence, as the *A. chromogenus*



types (*A. viridochromogenus*, *A. pheochromogenus*), *A. aureus*, *A. rutgersensis*, *A. violaceus ruber*. Jensen<sup>46</sup> found that *Act. griseus*, *Act. cellulosa*, *Act. violaceus*, *Act. bobili* and *Act. diastato-chromogenus* are the most common actinomyces in Danish soils.

*Importance of actinomyces in the soil.* Actinomyces take an active part in the decomposition of organic matter in the soil, both of a nitrogenous and non-nitrogenous nature. Some species are capable of decomposing cellulose very rapidly; under conditions favoring their development, as in neutral alkaline and arid soils or with insufficient moisture, actinomyces may play an important part in this process. Krainsky even divided all the actinomyces into two groups: (1) the macro-actinomyces, forming oval spores and large colonies on agar and not decomposing cellulose or only to a very limited extent; and (2) the micro-actinomyces, forming spherical spores and minute colonies on agar and decomposing cellulose rapidly with the formation of pigments. Macé<sup>47</sup> has shown that actinomyces decompose proteins into amino acids and ammonia; he suggested that they may bring about the formation of humus (ulmic acids) in the soil. Active protein decomposition by actinomyces has been recorded by a number of investigators.<sup>48</sup> In view of the fact that the amount of mycelium synthesized by this group of organisms is considerably smaller than that of fungi, only small amounts of nitrogen are assimilated and most of it is liberated free in the form of ammonia. Non-nitrogenous organic matter does not exert such a depressing effect upon ammonia accumulation as in the case of bacteria and other fungi.

The accumulation of "humus" in the soil is an index of the great resistance of this group of organic substances to decomposition by micro-organisms. Since this substance contains the larger part of the soil nitrogen, its decomposition is of great importance to soil fertility. Actinomyces seem to be among the very few organisms capable of attacking this resistant material and bring about its decomposition. Liming of soil and draining of swampy soil favors the development of actinomyces and also the decomposition of the soil organic matter. It is possible that a definite connection exists between these two phenomena. According to Fousek, an increase in plant growth is obtained by adding actinomyces mycelium to soil, due to the increased decomposition of the organic matter thus brought about.

<sup>46</sup> Jensen, H. L. *Soil Sci.* 30: 59-77. 1930

<sup>47</sup> Macé, E. *Compt. Rend. Acad. Sci.*, 141: 147. 1905.

<sup>48</sup> Fousek, 1913 (p. 289); Münter, 1914 (p. 286); Waksman, 1919 (p. 276).

All claims<sup>49</sup> that actinomyces are capable of fixing atmospheric nitrogen are based upon the observation that some of these organisms develop on media to which no combined form of nitrogen has been added. The fact that these organisms can thrive with mere traces of combined nitrogen and the possibility that combined forms of nitrogen in the atmosphere can also be utilized are not excluded. All positive claims reported so far are open to considerable criticism.

We find, among the actinomyces, organisms causing important plant diseases, of which potato scab, sugar beet and mangel beet scab,<sup>50</sup> as well as pox of sweet potatoes are known. There is considerable evidence that actinomyces may enter into certain associations with plants, as pointed out by Peklo,<sup>51</sup> who cultivated an organism, named *A. alni*, out of the nodules on the roots of *Alnus* and *Myrica*. Similar results were obtained by Lieske. In view of the fact that it has not as yet been possible to obtain the nodules by artificial inoculation, these results cannot be accepted as positive.

<sup>49</sup> Carter, E. G. and Greaves, J. E. *Soil Sci.* **26**: 179-198. 1928; Velich, V. *Vestn. Cesk. Akad. Zemed.* **5**: 584. 1929.

<sup>50</sup> Krüger, F. *Arb. Biol. Anst. L. Forstwirt.*, **4**: 254-318. 1905; Millard et al. (p. 293).

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## CHAPTER XIII

### SOIL PROTOZOA

The protozoa form numerically the most abundant group of the animal part of the soil population. It has been known since the work of Ehrenberg that the soil harbors a number of different protozoa, but the investigation of this subject has been greatly stimulated by the contributions of Russell and Hutchinson,<sup>1</sup> who suggested that the limitation of bacterial activities in soil is due to a factor of biological origin, probably the protozoa; this factor is less resistant than the soil bacteria and is readily destroyed by treatment of soil with heat or disinfectants. This treatment brings about a rapid development of the bacteria and the liberation of the combined nitrogen in a form available for plant growth, namely ammonia. The inference was that the soil protozoa, using the bacteria as food, limit bacterial activities in soil and, as a result of that, also limit soil fertility. On treating the soil with heat and disinfectants, the protozoa are destroyed, while the bacteria remain alive and begin to multiply very rapidly, their increased activities resulting in a greater liberation of nitrogen and an increase of plant growth.

Additional plausibility was given to this theory by suggestions made a few years previously and supported by a number of observations, that a similar function is performed by protozoa during the self-purification of water (i.e., the disappearance of pathogenic bacteria during storage). In fact, in view of the close similarity between many of the biochemical problems of water and sewage purification and those of the soil, the extensive investigations that have been made in the former fields are well worth careful consideration by the soil microbiologist.<sup>2</sup>

*General morphology of protozoa.* A detailed study of the morphology, classification, and physiology of protozoa is found in special texts de-

<sup>1</sup> Russell, E. J. and Hutchinson, H. B. Jour. Agr. Sci., 3: 111-144. 1909; 5: 152-221. 1913; Russell, E. J. Proc. Roy. Soc. B., 89: 76-82. 1915.

<sup>2</sup> Fehrs. Hyg. Rundsch., 16: 113-121. 1906; Buswell and Long. Illinois State water supply, Bul. 18, 1923.

voted to this subject.<sup>3</sup> Some of the information, necessary for a knowledge of the identification and study of activities of soil protozoa, is given here.

Protozoa are unicellular organisms, varying in size from a few microns to 4 to 5 cm. Some protozoa may also form colonies consisting of numerous individuals. The majority of species, particularly the soil forms, are microscopic and can be studied in detail only with the highest magnifications. The protoplasm is in a colloidal state and contains chromatic or nuclear substance, generally forming nuclei readily distinguishable from the protoplasmic body, which is either naked at the surface or enclosed by a cell membrane. Usually one or two nuclei are present, in some cases several of them; most infusoria contain a large macronucleus (vegetative functions and asexual division) and a small micronucleus (sexual reproduction). Contractile vacuoles, when present, are for the elimination of waste fluids or possibly for the adjustment of the osmotic pressure of the protoplasm (absent in marine forms). Green, yellow or brown chromatophores are present in the endoplasm of some Mastigophora. The most important constituents of the cell are the complex proteins, particularly nucleins and nucleo-proteins. In addition to these, there are always present in the living cell carbohydrates, lipoids and enzymes. There are also found in the protozoa undigested food particles, waste materials or foreign elements, which take no part in the physiology of the organism; algae and bacteria may often be present in the endoplasm, possibly as a result of symbiotic relationship. Many species are subject to the attacks of minute parasitic organisms, either in the nucleus or in the cytoplasm.

Reproduction is usually effected by fission, and, in the great majority of protozoa, a process of conjugation occurs at some stage in the life-cycle, the essential part in the process being fusion of the nuclear matter from distinct individuals. Locomotion is accomplished either by cilia, flagella, pseudopodia, or may be absent entirely, this serving as a basis for classification. Organs for the capture and assimilation of food may be present or entirely absent. The protozoa are classified on the basis of locomotion into:

<sup>3</sup> Bütschli, O. *Bronn's Klassen und Ordnungen des Thierreichs*. 1, 1883; Doflein, F. 1929 (p. XII); Minchin, E. A. *An introduction to the study of protozoa*. Arnold, London. 1912; Edmondson, C. H. *Proc. Davenport Acad. Sci.*, 12: 1-124. 1906. Schaeffer A. A. *Dept. Mar. Biol. Carnegie Inst., Washington* 24. 1926; Calkins, 1925 (p. XII).

1. Sarcodina or Rhizopoda, motility by means of pseudopodia, i.e., extensions (usually temporary) of the protoplasm of the cell body. The pseudopodia are either broad, blunt, finger-like or filiform, simple or branched. In some (*Heliozoa*), the ray-like pseudopodia are usually supported by axial filaments. Some Sarcodina are naked, while others form shells; these are composed of materials secreted by the animals themselves, as chitin, silica, calcium carbonate, or are constructed from foreign particles, as diatoms, sand grains, clay particles, etc. Some shells (chitinous) are delicate, transparent, while others are composed of distinct plates, arranged more or less regularly.

2. Mastigophora or Flagellata, motility by means of flagella. These flexible, whip-like processes are usually attached at one end of the body. Either one or more flagella may be present; when single the flagellum is usually directed forward and draws the body forward by its movement. When more than one flagellum is present, one or more may be directed backward. Some low flagellates can form pseudopodia.

3. Ciliata or Infusoria, motility by means of numerous cilia or short hair-like processes present during the entire existence of the protozoa or during their embryonic stage only. The cilia are either evenly distributed over the surface of the organisms or are restricted to certain regions. Large spine-like cirri or setae, or vibrating membranelles may be formed from fusion of cilia. Most are free swimming, some are attached by rigid or flexible stalks or pedicels.

4. Sporozoa, motility reduced by parasitism.

The soil forms are found among the first 3 general groups.

*Physiology of protozoa.* The physiology of the protozoa cannot be studied adequately, in a manner similar to higher organisms or to microscopic plants as fungi, bacteria and algae. This is due largely to the fact that they cannot be cultivated readily in pure cultures, free from bacteria. An approach in this direction has been made by the cultivation of protozoa with single species of bacteria.

Most of the protozoa are aerobic, obtaining their oxygen, necessary for oxidation purposes, from the air by absorbing it through the permeable membrane, without the aid of specialized respiratory organs. A few forms are anaerobic and many are semi-anaerobic. Oxidation is closely associated with nutrition and is followed by the excretion of the waste products, such as  $\text{CO}_2$ , urea and other products of metabolism. The optimum temperature for the development of protozoa is 18–22°C. Excessive heat kills the protozoa, but excessive cold does not injure them beyond retarding their vital activities.

The optimum reaction for the activities of protozoa was at first believed to be at the neutral point. Good growth was obtained, however, also in acid media.<sup>4</sup> The limiting acid and alkaline reaction values for the growth of *Paramoecium caudatum* were found to be at pH 5.0 and

<sup>4</sup> Vahlkampff. Arch. Protistenk., 5: 167. 1905.

9.0;<sup>5</sup> in other experiments *Paramoecium* was reported to develop normally at pH 6.4 to 8.0; beyond these limits, development is restricted and encystment may take place.

*Prowazekia* develops best at pH 6.2–6.6. Diminution of growth of protozoa in artificial media is not due to the exhaustion of nutrients, nor to an unfavorable change in reaction, but may be due to insufficient available oxygen and an insufficient removal of the volatile products of metabolism.<sup>6</sup> According to Darby,<sup>7</sup> the sequence of protozoan forms, as well as the question of encystment and excystment, are a result of the effect of the hydrogen-ion concentration; each protozoan has its own pH range. Various soil ciliates, flagellates and amoebae were found to be able to live and reproduce in artificial media even at pH 3.5–3.9 on the one hand and 9.75 on the other.<sup>8</sup> Testaceous rhizopods are most numerous in acid peat soils and are very scarce in alkaline soils.

Volatile antiseptics are believed to have a destructive effect upon protozoa. However, even 0.5 per cent concentration of CS<sub>2</sub> does not destroy the cysts of *Cercomonas crassicauda*, while 0.3 per cent or less will not even kill all the active forms. Different protozoa vary in their resistance to the disinfectant and different disinfectants vary in their effect.<sup>9</sup>

On the basis of their nutrition, the protozoa are divided into autotrophic (or holophytic forms which synthesize food using the energy of sunlight) and heterotrophic forms; these are subdivided into holozoic, when solid particles consisting largely of bacteria or other microorganisms are used as food, and saprophytic or saprozoic, when nourishment is absorbed from soluble organic substances by diffusion through the body surface. The great majority of protozoa are holozoic in their nutrition.

The autotrophic protozoa are usually found among the flagellates; they obtain their nutrition entirely from inorganic substances and from the CO<sub>2</sub> of the atmosphere. They are often spoken of as Phytoflagellates and may even be considered with the algae. They contain colored substances (chromophyll), which enables them to utilize the energy of light, similarly to higher plants. These colored substances are seldom

<sup>5</sup> Dale, D. 46: 129–140. 1913; Koffman, M. Arch. mikrosk. Anat. Entwickl.-lungs., 103: 168–181. 1924.

<sup>6</sup> Raabe, H. Bull. Soc. Chim. biol., 1: 383–400. 1925.

<sup>7</sup> Darby, H. H. Arch. Protistenk., 65: 1–37. 1929.

<sup>8</sup> Nasir cited by Sandon, 1927 (p. 314); Fine, M. S. Jour. Exp. Zool., 12. 1912.

<sup>9</sup> Dixon, A. Ann. Appl. Biol., 15: 110–119. 1928.



diffused throughout the cell, but are usually present in special bodies, the chromatophores. The most common representative of this group is the genus *Euglena*. In some cases, protozoa are found to live symbiotically with green algae. Such protozoa have been demonstrated among the amoebae and flagellates.

The holozoic protozoa, to which the majority of ciliates belong, use as food the various complex materials which form the constituents of living organisms (bacteria, algae). Some protozoa, like various amoebae, can feed on other protozoa, even on the larger ciliates.<sup>10</sup> The whole organism or part of it is taken into the body of the protozoan, where it is subjected to the chemical action of digestion, with the production of organic substances utilized by the protozoan as food. The process of nutrition takes place in three stages, the taking in of the food, its decomposition, and the excretion of the unutilizable part. Some protozoa, like *Actinophrys sol*, are omnivorous in nature; they eat readily ciliates and flagellates as well as various inanimate objects; they may even form colonies for the purpose of attacking large objects.<sup>11</sup> Some protozoa are actively cannibalistic in nature, ingesting and digesting their own kind.<sup>12</sup>

Digestion in all free-living protozoa is intracellular, by means of diastatic, proteolytic and other enzymes.<sup>13</sup> In some, if not in the majority of protozoa, there seems to exist a certain selection for the food, some species of bacteria being preferred to others. This may be due to the fact that some bacteria are digested more readily than others by the specific enzymes of the protozoa or to the formation by the particular bacteria of substances which are injurious to the protozoa.<sup>14</sup> Some protozoa are capable of digesting complex proteins, nitrogen being excreted as urea or as ammonia, as well as starches, and in some cases even cellulose.<sup>15</sup>

<sup>10</sup> Beers, C. D. *Science*, **64**: 90. 1926; *Brit. Jour. Exp. Biol.*, **1**: 325-341. 1924; Mast, S. O. and Root, F. M. *Jour. Exp. Zool.*, **21**: 33-49. 1916; Schaeffer, A. A. *Quart. Rev. Biol.*, **1**: 95-118. 1926.

<sup>11</sup> Looper, J. B. *Biol. Bull.*, **54**: 485-502. 1928.

<sup>12</sup> Dawson, J. A. *Jour. Exp. Zool.*, **29**: 473. 1919; *Proc. Soc. Exp. Biol. Med.* **26**: 335. 1929.

<sup>13</sup> Mouton, H. Thèse. Paris, 1902, Charaire; *Compt. Rend. Acad. Sci.*, **133**: 244. 1901. *Ann. Inst. Past.*, **16**: 457-509. 1902.

<sup>14</sup> Hargitt, G. T. and Fray, W. W. *Jour. Exp. Zool.*, **22**: 421-455. 1917; Phillips, R. L. *Ibid.*, **36**: 135-183. 1922.

<sup>15</sup> Wetherby, J. H. *Physiol. Zool.*, **2**: 375-394. 1929. Stolc, A. *Ztschr. wiss. Zool.*, **68**: 625-668. 1900; Cleveland, L. R. *Biol. Bul.*, **48**: 282-287. 1925.

Doflein divides the protozoa, which require complex organic substances as food, into 4 groups: those that feed on (1) bacteria, (2) on waste products, (3) on plants (diatoms and other algae), and (4) on small animals. Most forms take in mixed food, feeding on bacteria and waste products, but the fact that the different protozoa differ from one another in their feeding habits and that some species have considerable power of selecting their food, makes a more detailed knowledge of their food requirements a necessity for the satisfactory elucidation of their rôle in the soil economy.

When an infusion of hay, straw, or moss is prepared and allowed to incubate, bacterial development takes place immediately. This is soon followed by an abundant growth of various species of protozoa, including flagellates and amoebae, later followed by ciliates; these feed on the bacteria in the infusion. The protozoa come into the infusions from the air, where they are present in the form of cysts; as many as 13 species of protozoa have thus been demonstrated in the air.<sup>16</sup>

The soil amoebae and closely related forms select their food instead of ingesting all smaller organisms indiscriminately. Micrococci and bacteria are eaten most readily, bacilli less readily, while yeasts and actinomyces are not consumed at all.<sup>17</sup> Different species of bacteria have different feeding values for soil amoebae, both in respect to the rate of division and synthesis of protozoan protoplasm.<sup>18</sup> When an amoeba is once accustomed to feed on a certain bacterium, it will continue to select the particular organism in preference to others.<sup>19</sup>

Facts are on record concerning the ability of various protozoa to utilize soluble organic substances formed in the soil by bacterial action. This is true of flagellates and even of certain ciliates, which are said to have been cultivated free from bacteria, and to be able to derive their nutrients from various soluble organic and inorganic materials.<sup>20</sup> Under natural conditions, however, *Colpidium colpoda* may feed entirely by phagocytosis, although, under artificial conditions, they may be able to obtain their food from solution.<sup>21</sup> These results need further confirmation, the evidence submitted being insufficient to be accepted.

<sup>16</sup> Puschkarew, B. M. Arch. Protistenk., **28**: 323. 1913.

<sup>17</sup> Severtzoff, L. B. Centrbl. Bakt. I, Orig., **92**: 151-158. 1924.

<sup>18</sup> Cutler, D. W. and Crump, L. M. Brit. Jour. Exp. Biol., **5**: 155-165. 1927.

<sup>19</sup> Oehler, R. Arch. Prot., **37**: 175-190. 1916; **49**: 112-134. 1924.

<sup>20</sup> Thornton, H. G. and Smith, G. Proc. Roy. Soc. B., **88**: 151-165. 1914; Alexiev, A. G. Russ. Jour. Microb., **4**: 97-134. 1917; Peters, R. A. Jour. Physiol., **55**: 7-32. 1917.

<sup>21</sup> Lwoff, A. Compt. Rend. Acad. Sci., **176**: 928-930. 1923.

*Media for the cultivation of protozoa.* The media commonly employed in the cultivation of protozoa consist of complex organic substances. For the cultivation of chlorophyll-bearing protozoa, as well as many heterotrophic forms, purely synthetic media can be used.

For the growth of *Euglena*, a medium consisting of 0.5 gram peptone, 0.5 gram glucose, 0.2 gram citric acid, 0.2 gram  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05 gram  $\text{K}_2\text{HPO}_4$  in 100 parts of water has been suggested.<sup>22</sup> The concentration of citric acid and peptone may be doubled and 0.05 per cent  $\text{NH}_4\text{NO}_3$  may be added.

The green *Paramoecium bursaria* was cultivated<sup>23</sup> on a medium consisting of:

$\text{Ca}(\text{NO}_3)_2$ .....	0.20 gram	$\text{NaCl}$ .....	0.20 gram
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .....	0.02 gram	$\text{FeSO}_4$ .....	Trace
$\text{K}_2\text{HPO}_4$ .....	0.02 gram	Water.....	1000 cc.

Peters' glycerophosphate medium has been used successfully for the cultivation of ciliates. *Colpidium* and *Oicomonas* were grown on the following medium:<sup>24</sup>

Ammonium lactate....	0.1 gram	$\text{KCl}$ .....	0.3 gram
Glucose.....	0.4 gram	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .....	0.001 gram
$\text{NH}_4\text{Cl}$ .....	0.03 gram	$\text{CaCl}_2$ .....	0.02 gram
$\text{Na}_2\text{HPO}_4$ .....	0.01 gram		

The reaction is adjusted to pH 7.0-7.4. Phenol red may be added to the cultures to follow changes in the acidity and alkalinity during the growth of the culture. Giltay solution and a synthetic glycerol medium may also be employed.

The majority of the media used for the isolation of protozoa are based upon a previous development of bacteria, which, either alive or dead, may serve as food for the protozoa. Such substances, as soil, straw, hay, grass, lettuce, leaves, etc. cooked in water are favorable.

Thirty to 40 grams of hay are cooked in one liter of water for thirty minutes;<sup>25</sup> solution is filtered, made up to volume; to prepare a solid medium 15 grams of agar are added. 0.025 per cent meat extract in distilled water was found<sup>25</sup> to be favorable for *Paramoecium aurelia*. Other media containing one to two per cent of nutrose, somatose, or peptone and 1.5 per cent agar are also favorable. Martin and Lewin<sup>27</sup> used a medium prepared by boiling three lumps of horse manure in

<sup>22</sup> Zumstein. Jahrb. wiss. Bot., **34**: 149. 1900.

<sup>23</sup> Pringsheim, E. G. Biol. Centrbl., **35**: 375. 1915; Beitr. Allg. Bot., **2**: 88-137. 1921.

<sup>24</sup> Cutler, D. W. and Crump, L. M. Biochem. Jour., **17**: 174-186, 878-886. 1923; Abderhald. Handb. biol. Arb. Method. Abt. XI, T. 3. 1926.

<sup>25</sup> Further information on hay infusions is given by Woodruff, L. L. Jour. Exp. Zool., **12**: 206-264. 1912; Fine, M. S. Ibid., **12**: 265-289. 1912.

<sup>26</sup> Woodruff, L. L. Jour. Exp. Zool., **12**: 205. 1912; **14**: 575. 1913.

<sup>27</sup> Martin, C. H. and Lewin, K. R. Phil. Trans. Roy. Soc. B, **205**: 77-94. 1914; Jour. Agr. Sci., **7**: 106-119. 1915.

500 cc. water for  $1\frac{1}{2}$  hours, filtering through cloth and adding 6 grams of agar. A small amount of water or dilute albumin added to the culture plates to a depth of 2 mm. favored the growth of protozoa. Meat extract agar may also be used. Bacterial growth takes place from those cells which are transferred with the protozoa. In some cases, special bacterial cultures are inoculated. To prevent the accumulation of injurious by-products, the protozoan culture must be frequently transferred. A rapid development of bacteria may lead to the destruction of protozoa or may prevent their growth.<sup>28</sup> Media containing 0.3 to 0.5 gram Liebig's beef extract, 0.3 to 0.5 gram NaCl and 20 grams of agar in 1000 cc. of water have commonly been employed. Beijerinck<sup>29</sup> used yeasts for the cultivation of amoebae and ciliates; as a solid medium, a mixture of 20 parts of agar, 100 parts of beef bouillon and 900 parts distilled water of a neutral or slightly alkaline reaction may be employed.

The use of dead bacteria for the cultivation of amoebae has been suggested.<sup>30</sup> Amoebae and ciliates are capable<sup>31</sup> of assimilating pure cultures of bacteria, utilizing dead cells as well as small particles of protein material. Gram negative bacteria were preferred to gram-positive forms; young bacterial cultures in many cases were more assimilable than old cells. Successful cultures were obtained on bacteria smeared on the plate, then autoclaved at  $130^{\circ}$ , for 1 hour. *Azotobacter*<sup>32</sup> offers good food for amoebae, on a medium containing 10 grams of dextrin, 2 grams  $K_2HPO_4$ , 0.2 gram  $MgSO_4$ , 0.2 gram  $CaCO_3$ , 10 grams agar in 1000 cc. water. *Paramoecium* was also grown in pure cultures of bacteria.<sup>33</sup> Severtzoff<sup>34</sup> cultivated an amoeba on a pure culture of *Bact. coli*; she found that the bacterium was destroyed by a small quantity of chlorine, while the protozoan cysts were left unaffected. As soon as the chlorine was removed, the amoebae excysted and began to feed rapidly on the dead bacterial cells; when the food supply became exhausted, the protozoa encysted again.<sup>35</sup>

<sup>28</sup> Oehler, R. Centrbl. Bakt. I, Orig., **86**: 494-500. 1921.

<sup>29</sup> Beijerinck, M. W. Centrbl. Bakt. I, **19**: 257-267. 1896; **21**: 101-102. 1878; Mouton, 1902 (p. 302); Wasielewski, T. V. and Kühn. Zool. Jahrb. Anat., **38**: 253. 1914; Heidelberg. Akad. Wiss. Math. u. Naturw., **1**: 1-31. 1913.

<sup>30</sup> Tsujitani, J. Centrbl. Bakt. I, **24**: 666-670. 1898.

<sup>31</sup> Oehler, R. Arch. Protistenk., **37**: 175-190. 1916; **40**: 16. 1919; **41**: 34. 1920; **49**: 112-134. 1924; Gozony, L. Centrbl. Bakt. I, Orig., **84**: 565-566. 1920.

<sup>32</sup> Welch, M. W. Trans. Amer. Micr. Soc., **36**: 21-25. 1917.

<sup>33</sup> Hargitt, F. T. and Fray, W. W. Anat. Rec., Philadelphia, **11**: 516. 1916; Jour. Exp. Zool., **22**: 421. 1917.

<sup>34</sup> Severtzoff, 1924 (p. 303).

<sup>35</sup> Further information on the cultivation of amoebae is given by Schaeffer, A. A. Jour. Anim. Behavior, Cambridge, **7**: 220-258. 1917; Arndt, A. Centrbl. Bakt. I, Orig., **88**: 417. 1922; Chatton, E. and Chatton, M. Compt. Rend. Acad. Sci., **176**: 1262-1265. 1923; Cunningham, A. and Löhnis, F. Centrbl. Bakt. II,

Robertson<sup>36</sup> ascribed the autocatalytic phenomenon observed in the growth of protozoa to an accessory foodstuff, a soluble product of bacterial metabolism. According to Cutler it seems very uncertain that autocatalysis occurs in protozoan growth, but, if the curve is autocatalytic in nature, this is due to an increased food supply (bacteria) and not to an accessory food as Robertson suggested. Certain species of bacteria, like *Bact. fluorescens* and *Bact. coli*, may produce substances injurious to the development of the protozoa. This injurious effect may be due to the consumption of oxygen by the bacteria, or to a change in reaction brought about by bacterial growth.

*Isolation of pure cultures of protozoa.* By examining crude cultures of protozoa from time to time, it is found that there is usually in any given culture a more or less definite succession of animal forms. By selecting the time and method of culture, it is possible to isolate pure protozoan cultures. Several methods have been utilized<sup>37</sup> for the isolation of amoebae, the simplest of which consists in destroying the bacteria by means of heat or disinfectants (also 2 per cent HCl over night or 20 per cent Na<sub>2</sub>CO<sub>3</sub> for 3 days) in encysted protozoan cultures; the more resistant cysts survive. The use of the Barber pipette or Chambers' apparatus has also been suggested. Oehler inoculated first a dish containing agar or gelatin medium with a pure culture of a certain bacterium, then a mixed protozoan culture was inoculated into the center of the dish. After three days, the protozoa reached the edge of the dish. The process was repeated until finally a culture of protozoa was obtained free from any bacteria except the species inoculated into the dish.

A sample of the liquid containing the protozoa may be brought into a fine capillary tube, from which a single drop is ejected on a counting chamber (haemocytometer pattern) and immediately examined under the microscope. When a drop is obtained which contains a single organism, a drop of sterile medium or a culture of the specific bacterium is placed upon a clean cover slip which is then put on the chamber so that both drops coalesce. The chamber is then placed in a Petri dish lined with moist filter paper, and incubated. By projecting the image of the drop upon a screen, the multiplication of the protozoa can be followed.<sup>38</sup>

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39: 596-610. 1914; Killer, J. *Ibid.*, 37: 521-534. 1913; Volz, P. *Arch. Protistenk.*, 68: 349-408. 1929.

<sup>36</sup> Robertson, T. B. *Biochem. Jour.*, 15: 595-611, 612-619. 1921.

<sup>37</sup> Gordon, C. E. *Science*, 46: 212. 1917; Glaser, R. W. and Cosia, N. A. *Jour. Exp. Med.*, 51: 787-806. 1930.

<sup>38</sup> Cutler and Crump, 1923 (p. 304).

It is sometimes essential to obtain cultures of protozoa free from living bacteria. This has been done by Peters,<sup>39</sup> who isolated *Colpidium colpoda* from a hay infusion culture, in a drop of sterile medium, upon a sterile microscope slide.

By the use of a capillary pipette made of drawn-out glass tubing and fitted with a rubber bulb the organism was transferred, through several changes of sterile medium, upon sterile microscope slides. After about six washings, the organism was transferred to a fresh drop of sterile medium, which was placed in a two inch depression block and covered with a small sterile cover-glass, both of which had been previously sterilized by heat. By a process of trial and error, protozoa were finally obtained which divided well in the sterile medium. The culture was then transferred to a sterile tube plugged with cotton.

As a result of a series of studies, Peters demonstrated that glucose and ammonium lactate can serve as good sources of carbon and nitrogen for *Colpidium*. In addition to these, phosphates and chlorides, as well as potassium and magnesium are required. Amino acids can take the place of ammonium salts as sources of nitrogen. Carbon sources containing less than three carbon atoms in the molecule are not utilized; glycerate is used, but not lactate or citrate. Others<sup>40</sup> found, however, by the use of pure, bacteria-free, cultures of protozoa, that the latter cannot exist in culture solutions containing organic matter, but free from bacteria; these form the main food of the protozoa, which develop at the expense of the bacteria.

The concentration of the medium, reaction and temperature are of great importance in the cultivation of protozoa. A ciliate and a bacterium were cultivated<sup>41</sup> in a 0.1 per cent peptone solution, adjusted to pH 6.8, at 22° to 25°. After 24 hours, bacterial growth took place followed by that of protozoa. The latter could be separated from the bacteria electrolytically. Under the influence of the fall of potential, the protozoa travel to the cathode and the bacteria to the anode. This has to be repeated 6 times before cultures of protozoa free from bacteria are obtained. This procedure combined with the destruction of bacterial cells by heat were utilized by Oehler<sup>42</sup> for the purification of amoebae

<sup>39</sup> Peters, 1917 (p. 303). The results need confirmation and should not be considered as conclusive evidence; see also Paspart, A. K. Biol. Bull., 55: 113-120. 1928.

<sup>40</sup> Purdy, W. C. and Butterfield, C. T. Amer. Jour. Public Health, 8: 499-505. 1918; a recent review of the rôle of bacteria in the nutrition of the protozoa is given by Luck, J. M. et al. Quart. Rev. Biol., 6: 46-58. 1931.

<sup>41</sup> Amster. Centrbl. Bakt. I, Orig., 89: 166-168. 1922.

<sup>42</sup> Oehler, 1924 (p. 305).

and ciliates. By keeping dry cultures of protozoan cysts at 37° for 6 weeks, the bacterial vegetative cells were destroyed. When the dry cysts were placed in water and a culture of *Saccharomyces* added, the protozoa excysted. The yeasts can be killed at 60° for 24 hours. By the use of a cataphoresis apparatus and unpolarized electrodes in 0.65 per cent NaCl solution, the ciliates were found to travel to the cathode. However, the plate method was found to be best; the fact that protozoa travel faster than bacteria is utilized in the making of transfers. *Bact. fluorescens* was employed for repressing the accompanying bacteria.

*Staining of protozoa.* Protozoa should be studied both in unstained and stained<sup>43</sup> preparations.

<sup>43</sup> A detailed review of the fixing and staining of protozoa is given by Doflein, von Prowazek, Hargitt, G. W. Jour. Appl. Micros., 385-388. 1899; Goodey, T. Proc. Roy. Soc. (London) B, 84: 165-180. 1911; 88: 437-456. 1915; 89: 297-314. 1916; Bolles Lee. The microtome's Vademecum; Hartmann, M. Praktikum der Protozoologie. Fischer. Jena.

## PLATE XIV

### SOIL PROTOZOA

128. *Naegleria gruberi*: *a*, small organism (8 to 30 $\mu$ ) with one broad blunt pseudopodium or sometimes several blunt ones, and one subcentral nucleus. When it enflagellates the karyosome sends out a chromatic process *b*, which traverses the nuclear membrane, forms a marginal blepharoplast, and emerges as two long flagella (*c*, *d*). The body assumes a rigid asymmetrically curved shape and the organism swims away in the typical spiral course. When it exflagellates the flagella shorten, thicken and retreat into the cytoplasm and the blepharoplast returns to the karyosome within the nucleus (from Kofoid).

129. *Vahlkampfa soli*, a limax amoeba, from fresh fixed film (from Martin and Lewin).

130. *Amoeba cucumis*, a lamellipodian amoeba (from Martin and Lewin).

131. *Euglypha* sp., a thecamoeba (from Martin and Lewin).

132. Cyst of *Amoeba cucumis* (from Martin and Lewin).

133. *Colpoda steinii*,  $\times 800$  (from Goodey).

134. *Balantiophorus elongatus*,  $\times 800$  (from Goodey).

135. *Pleurotricha grandis* (?),  $\times 410$  (from Goodey).

136. *Gonostomum affine*,  $\times 510$  (from Goodey).

137. *Vorticella microstoma*, living stalked form,  $\times 410$  (from Goodey).

138. *V. microstoma*, free swimming, recently excysted form with aboral ciliary ring,  $\times 510$  (from Goodey).

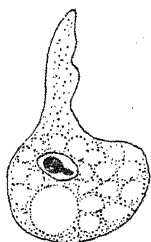
139. *Bodo caudatus* (from Martin and Lewin).

140. *Monas guttula* (from Fellers and Allison).

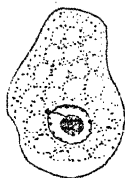
141. *Cercomonas crassicauda* (from Fellers and Allison).

142. *Bodo ovatus* (from Fellers and Allison).

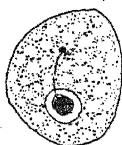
143. *Pleuromonas jaculans* (from Fellers and Allison).



A



B



C

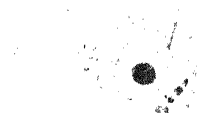


D

128



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Martin and Lewin<sup>44</sup> examined the active soil fauna in fresh films by using picric alcohol (50 per cent saturated solution picric acid in water + 50 per cent pure alcohol), or corrosive alcohol (50 per cent saturated solution of corrosive sublimate in water + 50 per cent pure alcohol). The soil is placed in a porcelain dish and enough of the fixative is poured through a funnel to the bottom of the soil layer until the soil is just covered: the dish is then slightly shaken. A film is formed which contains protozoa in a fixed and stained condition. By floating coverglasses on the surface of the liquid, the protozoa are removed and can be examined microscopically.

*Life history of protozoa.* The life history of a protozoan in the soil consists of a period of activity, when the animal moves, feeds, and reproduces, and a period of rest, when a thick wall is secreted around the body, and the cell (or cyst) becomes capable of resisting adverse conditions; the animal is distributed from place to place in the cyst state. When conditions become favorable, the wall is ruptured and the animal again becomes active. Sometimes, actual reproduction takes place within the cyst, as in *Colpoda steinii*. More seldom the cyst results from conjugation of two similar animals forming a large body known as the zygote.<sup>45</sup> The physiology of encystment and that of excystation are obscure points concerning which a considerable literature is growing up. Earlier writers regarded cyst formation as a direct response to unfavorable external conditions and excystation as occurring whenever a cyst found itself again in an environment suitable for active life. Experimental work has, however, failed to support this attractive theory and it appears that internal causes may play at least as great a part as external factors.<sup>46</sup>

Cysts are more resistant than active protozoa to the action of enzymes; also to chemicals,<sup>47</sup> the toxic order being  $CS_2$  > alcohol > acetone > benzol > ether > xylol > chloroform > carbon tetrachloride. The relative toxic effect of acids upon excystation is salicylic > butyric > oxalic > phosphoric > hydrochloric > sulfuric > acetic. Cysts and active organisms acclimatize to higher temperatures. The greater the degree of desiccation, the higher the resistance of cysts to higher temperatures and chemicals. The cyst of *Colpoda* consists of a carbohydrate, which is digested by an enzyme secreted by the enclosed organism during the process of excystation.<sup>48</sup> In other protozoa the

<sup>44</sup> Martin and Lewin, 1914-15 (p. 304).

<sup>45</sup> Martin, C. H. Proc. Roy. Soc. B, **85**: 393-400. 1912.

<sup>46</sup> Kofoid, C. A. Science, N. S., **57**: 397-408. 1923.

<sup>47</sup> Bodine, J. H. Jour. Exp. Zool., **37**: 115-125. 1923.

<sup>48</sup> Goodey, T. Proc. Roy. Soc. B, **86**: 427-439. 1913.

structure of the cyst wall is different, being siliceous in *Monas* and related flagellates. Excystation may take place by digestion of the wall, by its rupture, or by the emergence of the organism through a preformed pore which is a very characteristic structure in some forms.

Certain soil amoebae have also flagellate stages.<sup>49</sup> *Naegleria gruberi* (No. 128, Pl. XIV) is a soil amoeba found in California soils to a depth of over 20 feet. This amoeba has a biflagellate phase; it enflagellates and exflagellates rather quickly on slight provocation under the conditions of laboratory culture. It takes about 70 minutes for a culture of amoebae to change into the flagellate stage, while the reverse process is somewhat more prolonged and less uniform. The addition of water, of fresh culture medium (filtered and sterilized soil and manure infusions), or an excess of air tend to induce enflagellation, but exflagellation is less definite in response to opposite factors.<sup>50</sup>

*Occurrence of trophic and encysted protozoa in the soil.* The first detailed careful examination of the occurrence of active and inactive protozoa in the soil was carried out by Goodey<sup>51</sup> who concluded that ciliates are present in the soil only in an encysted condition and can, therefore, not function as a factor limiting bacterial activity in the soil. Martin<sup>52</sup> found that smaller amoebae and flagellates play the most important part in the phenomena of sick soils, while the limiting factor as regards the activity of protozoa in the soil is the average quantity of water. Subsequent investigations<sup>53</sup> demonstrated that a protozoan fauna normally occurs in the soil in a trophic state; this trophic fauna is most readily demonstrated in moist soil well supplied with organic matter, like heavily manured soils, sewage soils and especially greenhouse "sick" soils; the forms predominating in the soil are not necessarily the same as those that develop on artificial media (hay infusions) inoculated with soil.

A series of preparations of trophic amoebae, thecamoebae and flagellates were made by the methods described above. At first, flagellates were found<sup>54</sup> to be the only active forms in moist soils. It was shown

<sup>49</sup> Wilson, C. W. Univ. Cal. Publ. Zool., 16: 241-292. 1915. Kofoid, C. A. Science, N. S., 42: 937-940. 1915.

<sup>50</sup> A detailed discussion of natural history of protozoa is given by Doflein, F. Zool. Jahrb., 41: 1-112. 1919.

<sup>51</sup> Goodey, 1911-1915 (p. 308).

<sup>52</sup> Martin, C. H. Nature (London). 1913, 111.

<sup>53</sup> Martin and Lewin, 1914-1915 (p. 304); Goodey, 1916 (p. 308).

<sup>54</sup> Waksman, S. A. Soil Sci., 1: 135-152, 1916; 2: 363. 1916.

later<sup>55</sup> that flagellates, amoebae and thecamoebae are present in large numbers in a trophic condition in the soil; the fauna increases with an increase in the content of organic matter in the soil.

Various American investigators<sup>56</sup> believed that the protozoa exist in the soil mainly in a non-trophic state. Koch concluded that active protozoa do not exist in normal field soils or even in soils where the moisture content is somewhat above normal; in greenhouse soils containing much organic matter and a high moisture content, a few living protozoa were present. The protozoa become active in the soil, whenever the moisture content rises considerably above the normal; both moisture and organic matter were found to be the principal limiting factors in the development of protozoa in the soil. According to Cutler, there is an intimate mechanical association between the protozoa and the soil particles which depends on a mutual surface action; the capacity of various soil particles, such as sand and clay, for retaining these organisms is specific and constant. The difficulty of seeing living protozoa in the soil is due to the fact that the organisms rigidly adhere to the soil particles, and, up to a certain limit, they can be completely removed from a suspension by shaking for a few minutes with soil. Only in exceptional cases can organisms be dislodged sufficiently as to be recognized under the microscope. This is probably the reason why some investigators failed to observe living protozoa in the soil.

It has been established beyond any doubt that protozoa lead a trophic existence in the soil; this is especially true of the small flagellates. Even if most of them encyst after a continuous dry period, the first rain that brings the moisture content of the soil to optimum will lead to a rapid excystation of the protozoa and to a longer or shorter period of activity. Some protozoa will be found in an active state even in soils containing a low percentage of water. The flagellate *Cercomonas crassicauda* is capable of<sup>57</sup> excysting and reproducing in air-dried soils brought to one-sixth of their water-holding capacity. Various other common soil protozoa were found to behave normally in soil with one-half to one-third of its water-holding capacity, if previously dried. By the use of a capillary method, Koffman demonstrated the existence of tro-

<sup>55</sup> Crump, 1920 (p. 312); Cutler, D. W. Jour. Agr. Sci., 9: 430-444. 1919.

<sup>56</sup> Sherman, J. M. Jour. Bact., 1: 35, 165. 1916; Koch, G. P. Jour. Agr. Res., 4: 511-559. 1915; 5: 477-488. 1915; Soil Sci., 2: 163. 1916; Fellers, C. R. and Allison, F. E. Soil Sci., 9: 1-25. 1920.

<sup>57</sup> Cutler, D. W. and Dixon, A. Ann. Appl. Biol., 14: 247-254. 1927.

phic protozoa even in fairly dry soils; these consisted entirely of small flagellates (as *Monas termo*) and small rhizopods.<sup>58</sup>

A method was described by which it is possible to estimate the number of protozoa present in the soil both in a trophic and in a cyst condition.<sup>59</sup> The total number is first determined by the dilution method. A fresh portion of the soil is then treated with 2 per cent HCl (specific gravity 1.15) over night, whereby all active forms are killed. A second count by the dilution method gives the number of protozoa present in the soil in the form of cysts. The difference between the first and second counts gives the number of active protozoa. In this connection reference may be made to the work of Cunningham and Löhnis,<sup>60</sup> who found 60°C. to be the thermal death point of active protozoa (44° for flagellates, 48° for amoebae and 54° for ciliates), while 72°C. was found to kill the cysts. The temperature destructive to protozoa in soil is different from that of culture solutions. A temperature of 58°C. was used for distinguishing between cysts and active forms, but it was soon found that many cysts are also killed at that temperature. Drying reduces the number of protozoa, particularly ciliates and flagellates, amoebae preferring a somewhat drier soil.

The protozoa are widely distributed in the soil, comprising ciliates, flagellates, amoebae and thecamoebae. Some investigators reported an abundance of ciliates and flagellates and few amoebae;<sup>61</sup> others<sup>62</sup> found amoebae and thecamoebae most prevalent. The discrepancy may be due to the difference in methods used, especially in view of the sensitiveness of the latter two groups to the composition of the medium. The largest numbers of protozoa are present in the soil in spring, after the thawing of snow, or in summer, after heavy rainfall; only cysts are found in dry and semi moist soils.<sup>63</sup> The protozoan fauna of the naked sands of Central Asia was found<sup>64</sup> to be extremely poor, consisting only of

<sup>58</sup> Koffman, M. Centrbl. Bakt. II, 75: 28-45. 1928; Hino, J. Phytotechn. Inst. Miyazaki Coll. Agr. Nos. 12, 13, 29 (Ref. Proc. Int. Soc. Soil Si., 5: 166-168. 1930).

<sup>59</sup> Cutler, D. W. Jour. Agr. Sci., 10: 135-143. 1920.

<sup>60</sup> Cunningham and Löhnis, 1914 (p. 305); Jour. Agr. Sci., 7: 49-74. 1915.

<sup>61</sup> Feuilleteau and de Bruyn, W. K. H. Centrbl. Bakt. II, 56: 12-13. 1922.

<sup>62</sup> Crump, L. M. Jour. Agr. Sci., 10: 182-198. 1920; the occurrence of amoebae in soil is discussed further by Volz, P. Arch. Protistenk., 68: 349-408. 1909.

<sup>63</sup> Coppa, A. Staz. sper. Agrar. ital., 54: 181-213. 1921; Nowikoff, M. Die Bodenprotozoen und ihre Bedeutung für die Bodenkultur. Winter. Heidelberg, 1923.

<sup>64</sup> Brodsky, A. and Yankovskaya, A. Acta. Univ. Asiae. Mediae, 12: F. 6. 1929.

*Oicomonas mutabilis* and *Colpoda steinii*. Fixed sands, however, contain many more protozoa, namely 30,000 per gram in the 20-30 cm. horizon.

According to Crump, the protozoan fauna is largely confined to the top six inches of soil. In arid regions, especially in poor sandy soils, the protozoa are found in greatest abundance somewhat below the surface. Irrigation of arid soils stimulates considerably the development of numerous protozoa.<sup>65</sup> The amoebae are not influenced by variations in the water content and temperature of the soil and by the rainfall. The richer the soil is in organic matter the richer it is in protozoa, especially in amoebae and thecamoebae. A detailed study has been made of the presence of protozoa in peat soils,<sup>66</sup> in Egyptian,<sup>67</sup> Italian<sup>68</sup> and German soils,<sup>69</sup> in the soils of the United States,<sup>70</sup> in Russian,<sup>71</sup> Indian,<sup>72</sup> Swedish<sup>73</sup> and in English soils;<sup>74</sup> an abundant fauna was found in various samples of moss and soil from Spitzbergen and in soils of various South Sea and Atlantic islands (Tristan da Cunha, Gough Islands, etc.).<sup>75</sup>

*Classification and occurrence of protozoa in the soil.* The majority of soil protozoa are cosmopolitan, since the species found throughout the world are, with some exceptions, identical, although not all the species are found in every soil. Protozoa were demonstrated in large numbers in soil by earlier investigators, namely Ehrenberg,<sup>76</sup> Greef,<sup>77</sup> and Rosenberg-Lipinsky,<sup>78</sup> who considered them to be of importance in soil fer-

<sup>65</sup> Belayeva, X. V. Central Asiatic Melior. Inst., 7, 1929.

<sup>66</sup> Scheffelt, E. Mikrokosmos, 15: 113-118. 1922.

<sup>67</sup> Ross, R. and Thomson, D. Proc. Soc. Med. Sec. Epidem., 9: 33. 1916.

<sup>68</sup> Cauda, A. and Sangiorgi, G. Centrbl. Bakt. II, 42: 393-398. 1914.

<sup>69</sup> Wolff, 1912 (p. 314); Oehler, 1916-1919 (p. 305).

<sup>70</sup> Kopeloff, N., Lint, C. and Coleman, D. A. Soil Sci., 3: 197-269. 1917. Fellers and Allison, 1918 (p. 311); Sandon, H. Soil Sci., 25: 107-121. 1927.

<sup>71</sup> Yakimoff, M. L. and Zeren, S. Centrbl. Bakt. II, 63: 33-57. 1924; Nowikoff, 1923 (p. 312).

<sup>72</sup> Chauduri, H. Ann. Protist. 2: 41-60. 1929; Rao, H. S. M. Jour. Ind. Inst. Sci. 11A: 111-119. 1928.

<sup>73</sup> Koffman, M. Acta Zool. 7: 277-328. 1926.

<sup>74</sup> Goodey, 1911-1915 (p. 308); Martin and Lewin, 1914-1915 (p. 47); Cutler and Crump, 1920 (p. 49); Sandon, 1927 (p. 314).

<sup>75</sup> Sandon, H. Linn. Soc. Jour., 35: 449-475. 1924; Sandon, H. and Cutler, D. W. Ibid., 36: 1-12. 1924.

<sup>76</sup> Ehrenberg, C. G. Die fossilen Infusorien und lebendige Dammerde. 1837. Berlin; Die Infusoriensthierchen als vollkommene Organismen. 1839.

<sup>77</sup> Greef, R. Arch. micros. Anat., 2: 299-311. 1866.

<sup>78</sup> Rosenberg-Lipinsky, Alb. v. Der praktische Ackerbau (etc.), 2: 27. 1869. Breslau.

tility. In more recent contributions, a study has been made of the influence of environmental conditions upon the occurrence of protozoa.

Fellers and Allison<sup>79</sup> demonstrated the presence of seventeen species of rhizopods, thirty-four flagellates, and fifty-one ciliates in New Jersey soils, fertile soils containing more species and greater numbers of protozoa than infertile soils. They concluded that the soil microfauna consists principally of small, hardy protozoa able to withstand, by means of encystation or otherwise, such extremes of heat and cold, desiccation, aeration, etc., as are natural to their life in the soil. Practically all species identified from the soil have been found also in fresh water lakes, ponds, pools and streams of New Jersey, but not in the same relative abundance, while several of the most common plankton organisms are rarely found in the soil.

Cutler and associates found six species of protozoa occurring constantly in the soil in sufficient numbers to admit the application of statistical methods to the results. These are: (1) *Dimastigamoeba gruberi*, (2) a small limax amoeba, (3) *Heteromita* sp. resembling *Bodo repens*, (4) a small soil flagellate, 3 to 6 by 2 to 3 $\mu$ ; (5) *Cercomonas* sp. and (6) *Oicomonas termo*. Sandon found the following average number of species of protozoa in 107 soils examined: 7.2 flagellates, 3.4 ciliates, 2.45 amoebae and 2.0 testaceous rhizopods. While some species grew in all media employed, other forms developed only upon certain specific media.

In all Sandon recorded 250 species of protozoa, some of which were observed in every soil, often in very large numbers. The flagellates *Heteromita globosus*, *Oicomonas termo* and *Cercomonas* sp., the ciliates *Colpoda cucullus* and *C. steinii*, and the limax amoebae *Näegleria gruberi* and *Hartmanella hyalina* were most common and most abundant. Most protozoa found in the soil are also present in various other habitats, such as standing and flowing fresh waters, sea water and plankton; a few are found only in the soil. The extreme climate of arctic land is not in itself an obstacle to the abundant development of protozoa, provided the soil is well manured and in good condition; however, peat soils

<sup>79</sup> Wolff, M. Mitt. Kaiser Wilhelm Inst. Landw. Bromberg., 1: 382-401. 1909; Centrbl. Bakt. II, 33: 314-320. 1912; Martin and Lewin, 1914-1915 (p. 47); Fellers and Allison, 1920 (p. 311); Cutler, Crump and Sandon, 1922 (p. 29); Fantam, H. B. and Taylor, E. So. African Jour. Sci., 18: 373-393. 1922; 19: 340-371. 1922; 20: 437-492. 1923; 21: 445-479. 1924; Sandon, 1924 (p. 313); Sandon and Cutler, 1924 (p. 313); Sandon, H. The composition and distribution of the protozoan fauna of the soil. Oliver and Boyd. Edinburgh and London. 1927.

are decidedly unfavorable for the development of protozoa except for the testaceous forms. Fantham and Taylor found 1 to 22 species in each of a series of South African soils; the flagellates were largest in total numbers, while the ciliates showed the largest number of species; dark, heavy, humus rich soils contained more protozoa than sandy soils; the reaction of the soil had no effect upon the protozoan fauna. A close positive connection has also been observed (Cutler et al.) between the numbers of protozoan species and bacteria in the soil.

The protozoa reported to have been found in the soil can be classified as follows:<sup>80</sup>

#### A. SARCODINA<sup>81</sup>

##### I. ACTINOPODA, pseudopodia with axial filaments.

*Heliozoa*, spherical, with fine radiating pseudopodia, with stiff axial rods; endoplasm surrounded by vacuolated ectoplasm. The following representatives of this group have been found in the soil: *Actinophrys sol* (F, I, K, J, O), *Raphidiophrys* (F), *Acanthocystis aculeata* (I), *Clathrulina elegans* (F).

##### II. RHIZOPODA, pseudopodia without axial filaments.

1. PROTEOMYXA, pseudopodia fine and radiating, often anastomosing or forming a network. Among the soil forms belonging to this group are *Biomyxa vagans* (J), *Nuclearia simplex* (A, I, J, L, M), *Gephyramoeba delicatula* (C and J), *Leptomyxa reticulata* and *L. flabellata* (C), *Vampyrella laterita* (F).

2. AMOEBOEA, naked or with chitinous shells (tests).

(a) *Amoebida*, naked rhizopoda, without any shells or supporting structures; pseudopodia blunt or pointed but

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<sup>80</sup> The following letters can be used to designate the names of the investigators, who have demonstrated the presence of the specific protozoa in the soil: A—Wolff in Germany, B—Francé in Germany, C—Goodey in England, D—Martin or Martin and Lewin in England, E—Waksman in New Jersey, U. S. A., F—Fellers and Allison in New Jersey, U. S. A., G—Cutler, Sandon and Crump in England, H—Cutler and Sandon in Spitzbergen soils, etc., I—Fantham and associates in South African soils, J—Sandon working with soils collected throughout the world, K—Yakimoff and Zérén in Russia, L—Perey (Ann. Sci. Agron. 39: 333-352. 1923) in France, M—Allison (Soil Sci. 18: 339-352. 1924) in England using American soils, N—Nowikoff in Russia, O—Koffman in Sweden, P—Sandon in the United States.

<sup>81</sup> For the identification of this group, consult, in addition to the general treatises and papers, also Edmondson, C. H. In Ward and Whipple's Fresh-water biology. 1918, p. 210; Leidy, J. U. S. Geol. Surv., 12: 324. 1879; Cash, J., and Wailes, C. H. The British freshwater rhizopoda and heliozoa. Vols. 1-5, 1905-1921. Roy. Soc. London; Penard, E. La faune rhizopodique du bassin de Léman. Genève. 1902; Poche, F. Arch. Protistenk., 30: 251-310. 1913; Ghosh, E. Jour. Roy. Micr. Soc., 272: 327-329. 1925; Sandon, 1927 (p. 314).



never filamentous; may be reduced to wave-like expansions of protoplasm.

- (a') *Amoeba limax* group. Small amoebae with single rounded pseudopodium; sometimes several finger-like pseudopodia formed simultaneously.

*Naegleria gruberi*, one of most common soil protozoa recorded by Wilson, F, G, O and P. *Hartmannella hyalina* is also very abundant in the soil. *Amoeba (Hyalodiscus) guttula* was found by A, B, F, I, J, K and P.

- (b') *A. verrucosa* group. Pseudopodia in the form of ridges or folds of the ectoplasmal pellicle. *A. verrucosa* (*A. terricola*?) was found in the soil by A, B, C, I, J, K, N and P; *A. diploidea* by D, J, P; *A. striata* by B, J and P.
- (c') *A. lamellipodia* group, similar to previous group, with less strongly developed pellicle, including: *A. glebae* (J), *A. actinophora* (J), *A. gobanniensis* (D).
- (d') *A. proteus* group. Large amoebae, changeable in shape, with numerous long, cylindrical pseudopodia. Representatives of this group were found in a number of soils by B, I, J, O, P.
- (e') Various other amoebae have been found in the soil by different investigators, such as *A. radiosa* (F, J, K, O, P), *A. albida* (J, P), *A. alveolata* (O), *A. gorgonia* (O), *A. vespertilio* (O), etc.
- (b) *Testacea*, rhizopods with shells (tests), into which the whole body can be withdrawn.
- (a') *Arcellidae*, shells chitinous, pseudopodia lobose or simply branched. *Arcella vulgaris* was found in the soil by A, B, C, F, I, J, N, O; *A. discoides* by A and J; *Pseudochlamys patella* by B; *Corycia flava* by B; *Diffugiella* by B and J; *Hyalosphenia elegans*, *H. cuneata*, *H. pupilis* and *H. tinctoria* by B; *H. minuta* by J.
- (b') *Diffugiidae*, chitinous shells covered by foreign bodies. Representatives of this group found in the soil are *Diffugia pyriformis* (B, F, I, J, O), *D. globulus* (B, C, F, I, J, P), *D. penardi* (*D. fallax*) (J), *D. lucida* and *D. craterella* (B), *D. urceolata* (J), *D. lobostoma* and *D. arcula* (B, J), *D. constricta* (B, J), *Centropropyxis aculeata* and *C. laevigata* (J), *Phryganella acropodia* and *P. nidulus* (B), *Heleopera petricola*, *H. picta*, *H. rosea* and *H. sylvatica* (B).
- (c') *Euglyphidae*, chitinous shells with plates made by organism, including *Euglypha tuberculata* (J), *E. mucronata* (B), *E. bryophila* (J), *E. strigosa* (B and J), *E. globosa* (O), *E. rotunda* (J), *E. laevis*,

*E. ciliata* (B and J), *Placocysta spinosa* (B), *Nebela collaris* and *N. lageniformis* (B, J), *Quadrula symmetrica* and *Q. globulosa* (B), *Q. irregularis* (J), *Assulina muscorum* (B and J), *A. seminulum* (B), *Sphenoderia lenta* (B), *S. fissirostris* (J), *S. dentata* (B, J), *Campascus* sp. (B), *Trinema enchelys* (A, B, C, J), *T. lineare* (B, J, P) and *T. complanatum* (B, J), *Corythion dubium* (B, J).

- (d') *Gromiidae*, membranous shells, pseudopodia-reticulate, forming a network, including *Lecythium* (*Pamphagus*) *hyalinum* (Syn. *Chlamydothrys stercorea*) found by A, B, C, D, F, J, K, L, P; *L. mutabile* by B and F; *Pseudodiffugia gracilis* by B; *Allogromia fluvialis* (*Gromia terricola*) by Müller,<sup>82</sup> A and J; *Microgromia socialis* by F, J, K and O; *Diplophrys archeri* by F.

B. **MASTIGOPHORA.**<sup>83</sup> The soil flagellates are found largely in the following groups.

I. **PANTOSTOMATINAE.** Flagellates naked, colorless; food ingested, usually by means of pseudopodia, at all points of their surface; the organisms in this group possess one or more flagella and are usually more or less amoeboid.

*Actinomonas mirabilis* was found by J in 11 soils collected from various parts of the world. *Cercomonas crassicauda* and *C. longicauda*, are very common soil protozoa (F, G, J, K, O). *Cercobodo* was found by J to be represented in the soil by several species. *Mastigamoeba* and *Mastigella*, comprising organisms which closely resemble amoebae but possess a single flagellum, directed forward; the flagellum is connected with the nucleus in the case of the former, but not of the latter. *Mastigamoebae* have been found in the soil by F, I, J, L, N, O.

II. **PROTOMASTIGINAE.** Small flagellates, usually more or less amoeboid and having a fine periplast. Food taken in at one point, no chromatophores. Pseudopodia when present never acting as organs of locomotion. These include *Codonosiga botrytis* (C), *Monosiga ovata* (F and J), *Salpingoeca convallaria* (A), *S. amphoridium* (O), *Phalansterium solitarium* (J in 56 out of 148 soils examined), *Bodo* (*Prowazekia*) *caudatus* (A, D, J, O), *B. edax* (Kühn,<sup>84</sup> G, J, K, N, P), *B. saltans* (A, C, F, J, N, P), *Bodo terricola* (D and others). A few

<sup>82</sup> Müller, P. E. Studien über die natürlichen Humusformen. Berlin. 1887.

<sup>83</sup> Klebs, G. Ztschr. wissensch. Zool., 55, 1893; Lemmermann's Algen I. Flagellaten. In Kryptogamenflora der Mark Brandenburg und angrenz. Gebiete. v. 3; Pascher, A., and Lemmermann, E. Flagellatae in "Die Süßwasserflora und Fauna Deutschlands." H. 12, Jena. 1913-1914; Senn, G. Flagellata, in Engler and Prantl's "Die natürlichen Pflanzenfamilien." Bd. I, T. 1, 1900; Conn, H. W. and Edmondson, C. H. Ward and Whipple's Fresh-water biology. 1918, p. 238. Keys for identification of soil flagellates, Sandon, 1927 (p. 314).

<sup>84</sup> Kühn, A. Arch. Prot., 35: 212-254. 1915.

other species of *Bodo* were reported in the soil by F, J, K and O. *Colponema symmetrica* (J), *Dinomonas vorax* (D), *Heteromita compressus* (J), *H. globosa* and *H. lens* are among the most common soil protozoa; these and *H. obovata*, *H. ovata*, *H. repens* were found in the different soils by H and J; some species of this genus were also recorded by A, E, F. *Phyllomitus undulans* by A, E and J, *P. amylophagus* found by F, G, J, P, *Pleuromonas jaculans* by A, E, F, I, *P. Sainouron mikroteron* was found to be common in Rothamsted soils by J and also in 45 other soils, found also by L and M; *Allantoin tachyploon* was found in the soil by J and M; *Phyllomonas contorta* by A; *Proleptomonas faecicola* is the only member of the Trypanosomaceae found by J as free-living in the soil; later *Dimastigella tripaniformis* was found (P) in American soils. *Spiromonas angusta* was found by A, E, G, J and Cunningham and Löhnis. *Spongomonas* is common in the soil (J), while *Cladomonas* was found in a Spitzbergen soil by J. *Tetramitus rostratus* was found by J and M, *T. spiralis* by C, J, L and M, *T. pyriformis* by J. F and K also recorded the presence of species of *Tetramitus* in the soil. *Hexamitus inflatus* was found by F, *Spiromonema multiciliatum* by C and J.

- III. CHRYSOMONADINAE. Small flagellates; when not possessing chromatophores, resemble the Protomastiginae. Cuticle generally present, but is thin and does not prevent them from becoming amoeboid; 1 or 2 flagella. Cysts endogenous, wall being more or less impregnated with silica. This group includes the following forms found in the soil: *Oicomonas termo* (D, F, G, J, K, O), *O. granulata* (K), *Chrysamoeba radians* (I), *Mallomonas* (E), *Monas guttula* (A, E, F, J, K, N, O, Koch, Cunningham and Löhnis), *M. vivipara* (E, F, N), *Cephalothamnion cyclopum* (J), *Physomonas elongata* (F, O), *Poly-pseudopodius bacterioides* (D).
- IV. CRYPTOMONADINAE. Small forms, with two flagella, usually equal, arising behind the anterior end in a hollow which is usually the opening of a funnel running deep into the interior of the cell. Egg-shaped and more or less flattened; body enclosed in membrane and not amoeboid. One or two simple contractile vacuoles at anterior end. These include *Chilomonas paramoecium* (A, F, I, J, K), *Cryptomonas* (F, J, K), *Cyathomonas truncata* (Cunningham and Löhnis, O), *Rhodomonas* (I).
- V. EUGLINIDAE,<sup>85</sup> characterized by a complicated vacuole system situated at anterior end and consisting of one or more accessory vacuoles which, in contracting, empty their contents into a large main vacuole or reservoir, which communicates with the cytopharynx. Mostly with green chromatophores, enclosed in a membrane and with 1 or 2 flagella. *Euglena acus* was found in the soil by F and K, *E. deses*, *E. oxyuris* and *E. spirogyra* by I, *E. velata* by B, *E. viridis* by A, E, F,

<sup>85</sup> For a detailed study of the morphology and physiology of this group, see Günther, F. Arch. Protistenk., 60: 511-590. 1928.

I, N, *Eutreptia viridis* by F, *Phacus longicauda* by F and I, *Ph. pyrum* by F and K, *Trachelomonas volvocina* and *Cryptoglena pigra* by F. Species of *Astasia* were found in the soil by D, F, N. *Distigma (Astasia) proteus* by B and K. *Clostenema (Sphenomonas) socialis* by F, *Menoidium* by J, *Petalomonas angusta* by J, *P. medio-canellata* by B and F, *P. pleurosigma* by I, *Scytomonas pusilla* by D, L and J, *Peranema trichorophorum* by B, F, I, K, *Urceolus cyclostomus* by K, *Anisonema minus* by J, *Entosiphon sulcatum* by C, I and J, *Heteronema acus* by F and N.

VI. PHYTOMONADINAE. Solitary or in colonies, enclosed in a cellulose wall; chlorophyll and stigma nearly always present; 1 or more simple contractile vacuoles at anterior end. *Chlamydomonas* sp. was found commonly in the soil by A, E, F, K, *Polytoma uwellia* by A, K and J, *Chlorogonium euchlorum* by J and K, *Pandorina morum* by A.

VII. DINOFLAGELLATA are enclosed in a rigid lorica and possess 2 flagella, one of which lies in a transverse groove and moves with an undulating motion and the other lies in a longitudinal groove and is trailed behind. Only one form, *Glenodinium pulvisculus*, has been found in the soil by I.

The last 5 orders are among the *Phytoflagellata*, the typical members of which possess chromatophores; colorless species are also found in all orders and it is these which are largely found in the soil.

C. INFUSORIA (CILIATA).<sup>88</sup> The common soil ciliates are found in the following groups:

I. HOLOTRICHA, body uniformly covered with cilia; these are similar or slightly lengthened about the mouth; no adoral spiral zone.

Various species of *Holophrya* were found in a number of soils by A, B, F, J, K, P; *Urotricha farcta* by N, P, O; *U. agile* by F; *Enchelys* is common in the soil, having been found by A, C, E, F, J, K, O, Koch, Cunningham and Löhnis; *Spathidium spatula* by C and K; *Lacrymaria* sp. by I and N; *Prorodon teres* by A, F and K; *P. ovum* by A, E, I and Koch; *Choenia* sp. by J; *Coleps hirtus* by I and K; *Mesodinium* sp. by F; *Amphileptus cygnus* and *A. gigas* by I; *Lionotus fascicola* by F and I; *Loxophyllum flexilis* and *L. rostratum* by I; *Dileptus* by F, J and K; *Nassula elegans* by A, E and I; *Chilodon cucullulus* by F, I, O; *C. megalotrocha* by F; *C. uncinatas* by J and K, and *Trochilia palustris* by A, J, P.

*Uronema marina* was found in the soil by A, F, J and K; *Glaucoma scintillans* and *G. pyriformis* by A, O; *Colpidium colpoda* by A, E and K and others, this being one of the most common soil ciliates. *C. striatum* was found by F and I. *Colpoda cucullus* and *C. steinii*, two of the most common soil ciliates, were recorded by most investigators on soil protozoa. *C. maupasii* was found in the soil by F and J. F also recorded the presence in the soil of *C. campyla*,

<sup>88</sup> Conn and Edmondson, Ward and Whipple's "Fresh-water biology;" Stokes, A. C. Jour. Trenton Nat. Hist. Soc., 1: 71-344. 1888; Roux, J. Genève, 1901.

*C. flavicans*, *C. helia* and *C. saprophila*. Various species of *Paramecium* have been found in the soil, although Sandon records the complete absence of this group in English and foreign soils. *P. aurelia* was found by I and N, *P. bursaria* by I, *P. caudatum* by F, I and N, *P. putrinum* by A and I, *P. trichium* by F, etc., *Pleuronema chrysalis* by A, F and I. Other species of *Pleuronema* were found by E, J and K. *Cyclidium glaucoma* was found by F, J, K, O; *Balantiophorus elongatus* by C, J and others, *B. minutus* by A, C, J, O, P; *Lembus pusillus* by F.

- II. **HETEROTRICHA.** Body uniformly covered with cilia, forming cirri or stout cilia in spiral adoral zone; undulating membrane often inside mouth. The following organisms belonging to this group were found in the soil: *Blepharisma ovata* by F, *B. laterita* by J, O; *Metopus sigmoides* and *Metapides acuminata* by F; *Spirostomum ambiguum* by I, *Condylostoma* sp. by Koch.
- III. **OLIGOTRICHA.** Spherical or conical, with adoral zone often forming a closed ring; cilia usually absent from other parts of body. The following forms were recorded as present in the soil: *Strombidium* sp. by E, F and I; *Halteria grandinella* by A, F, I, K, O. Other species of *Halteria* were found by B, E, J, N, P.
- IV. **HYPOTRICHA.** Body flattened dorso-ventrally; cilia often fused to form larger appendages or cirri, usually limited to ventral surface; adoral zone of membranellae. This group is represented in the soil by *Urostyla grandis* (C, K), *Stichotricha secunda* (F), *Uroleptus musculus* (A, F, K and Koch), *U. mobilis* (J), *U. piscis* (I, J, O), *U. dispar* (F, I, N), *Onychodromus grandis* (J and other investigators), *Gastrostyla steinii* (C, J, O), *Gonostomum* (*Plagiotricha*) *affine* (C, J, K, L, M, O), *Oxytricha fallax* (J, O), *O. bifaria* (F), *O. pellionella* (Cunningham and Löhnis, F, I, J, O); other species of *Oxytricha* were also found by different investigators of soil protozoa. *Pleurotricha lanceolata* and *P. grandis* (C, J, O), *Stylylonychia mytilus* (B, F, I), *S. pustulata* (F, K, O), *Euplotes carinata* (F, J), *E. charon* (A, F, J, O), *E. harpa* and *E. patella* (I), *Aspidisca costata* (F, I, K) and *A. lyncaster* (K).
- V. **PERITRICHA.** Body cup-like or cylindrical, often stalked, of a sedentary habit; cilia usually limited to adoral zone, the membranellae leading down to a vestibule, into which pharynx and contractile vacuoles open; a posterior ring of cilia may be temporarily present. This group is represented in the soil by *Vorticella microstoma* (C, F, J, K, N, O, P), *V. citrina* and *V. globularia* (F), *V. nebulifera* (K, N), *V. putrina* (A, F); other species of *Vorticella* have also been found by different other investigators. *Epistylis coarctata* (C), *Cothurnia doliolium* (B), *Vaginicola terricola* (B).

*Importance of protozoa in the soil.* No definite evidence has as yet been submitted as to the actual rôle of protozoa in the soil. We know, on the one hand, that certain groups of protozoa at least, particularly

the ciliates and amoebae, are not only capable of ingesting bacteria, but some actually use this sort of food exclusively.<sup>87</sup> By determining the number of amoebae and bacteria in the soil, at daily intervals, an inverse relationship has been obtained between these two groups of organisms. Cutler<sup>88</sup> purified cultures of an amoeba and a flagellate so that they contained only three species of common soil bacteria. These were isolated free from protozoa, and suspensions were prepared of the bacteria alone and bacteria + protozoa, the latter in an encysted condition. These suspensions were sprayed on by a fine nozzle upon 100 grams of sterile soil in large sterile Petri dishes. The protozoa added per 1 gram of soil were: 25,000 for the *Dimastigamoeba gruberi*, 20,000 for *Cercomonas crassicauda* and 11 to 13 millions of bacteria. At the end of 15 days, the numbers of protozoa were: 230,000 amoebae and 420,000 flagellates per gram. The bacteria, in the protozoa free culture, reached a maximum of 214.4 millions in 6 days, then diminished to 169.2 millions in 15 days (21 per cent decrease); in the presence of the amoebae, the maximum of bacteria (178.4 millions) was attained in 3 to 5 days, then the decrease was more rapid, falling down to 72.8 millions in 15 days (59 per cent decrease); in the presence of flagellates the maximum of 103 millions was reached in 7 days, dropping to 88 millions in 15 days (14.5 per cent decrease).

Goodey<sup>89</sup> has previously shown that amoebae of the limax group and other larger forms can lead an active existence in the soil and exert a depressing effect upon bacterial numbers. He suggested the probability that a certain point must be reached in protozoan development before the depression in bacterial numbers is caused; this number seems to be about 30,000 cells of *Amoeba limax* per gram of soil.

On the other hand, certain suggestions have been made that protozoa can live in the absence of bacteria. Breal<sup>90</sup> (1896) believed that Colpoda is active in the decomposition of plant constituents of the soil with the production of ammonia. Other investigators<sup>91</sup> claimed that the protozoa play an important rôle in the decomposition of organic matter in the soil. A number of protozoa are found<sup>92</sup> to be saprophytic in nature

<sup>87</sup> Huntentüller, O. Arch. Hyg., 54: 89-100. 1905; Calkins, 1926 (p. XII). Purdy and Butterfield, 1918 (p. 307); Cunningham, A. Jour. Agr. Sci., 7: 49-74; 1915; Cutler and Crump, 1920 (p. 49).

<sup>88</sup> Cutler, D. W. Ann. Appl. Biol., 10: 137-141. 1923.

<sup>89</sup> Goodey, T. Proc. Roy. Soc., 89: 297-314. 1916.

<sup>90</sup> Breal, E. Ann. Agron. 22: 362-375. 1896.

<sup>91</sup> Müller, 1887 (p. 667), p. 15, 56, 167; Hiltner, L. Jahresb. Ver. angew. Bot. for 1907, 5: 200-222. 1908.

<sup>92</sup> Minchin, 1912 (p. 299).

and capable of obtaining their food by absorption. The same may be true of the soil flagellates, as evidenced by the work of Thornton and Smith and Alexeiev, and even ciliates, as shown by Peters. In most instances, of course, no direct evidence has been submitted to prove that the protozoa take an active part in the decomposition of organic matter. Goodey has shown that when various protozoa are added to the soil, bacterial activity is not limited, as seen in table 22. This seems to be contrary to the latter results of Goodey<sup>93</sup> mentioned previously; however, they are explained by the fact that a soil treated with an anti-septic does not afford a suitable medium for the development of protozoa. The drop in the numbers of bacteria follows the exhaustion of available plant food in the soil.

TABLE 22  
*Bacteria in millions per gram*

	INCUBATION, DAYS						
	Start	32	60	93	151	208	487
Untreated.....	14.4	10.3	13	11.4	12	8	12
Toluene treated.....	9.2	73.0	60	61.0	40	49	56
Toluene + untreated soil.....	11.3	49.0	61	43.0	19	45	48
Toluene + ciliates.....	4.5	371.0	292	296.0	56	64	73
Toluene + amoebae.....	3.0	285.0	185	141.0	74	90	57
Toluene + flagellates.....	27.0	247.0	214	227.0	196	104	113
Toluene + bacteria.....	2.3	500.0	341	311.0	181	151	150

Few attempts were made to demonstrate whether protozoa actually injure important soil biological processes. Nasir<sup>94</sup> determined the influence of the presence of protozoa (*Colpidium colpoda*) upon the fixation of nitrogen by *Azotobacter* in mannitol cultures, both in solution and in sand. In 31 experiments out of 36, the presence of protozoa resulted in an increase in the amount of nitrogen fixed by *Azotobacter*. The feeding action of protozoa upon *Azotobacter* seems to stimulate the further development of the latter and thus maintain its nitrogen-fixing efficiency for a longer period<sup>95</sup> (Fig. 10). Only small quantities of soluble nitrogen were found<sup>96</sup> in pure cultures of *Azotobacter*, produced as a

<sup>93</sup> Goodey, 1915 (p. 308).

<sup>94</sup> Nasir, S. M. Ann. Appl. Biol., 10: 122-123. 1923.

<sup>95</sup> Cutler, D. W. and Bal, D. V. Ann. Appl. Biol. 13: 516-534. 1926; Fedorowa-Winogradowa, T. Centrbl. Bakt. II, 72: 374-379. 1927; 74: 14-22. 1928.

<sup>96</sup> Moler, T. Bot. Notiser. 1915, 163-175. (Centrbl. Bact. II, 47: 635-636. 1917).

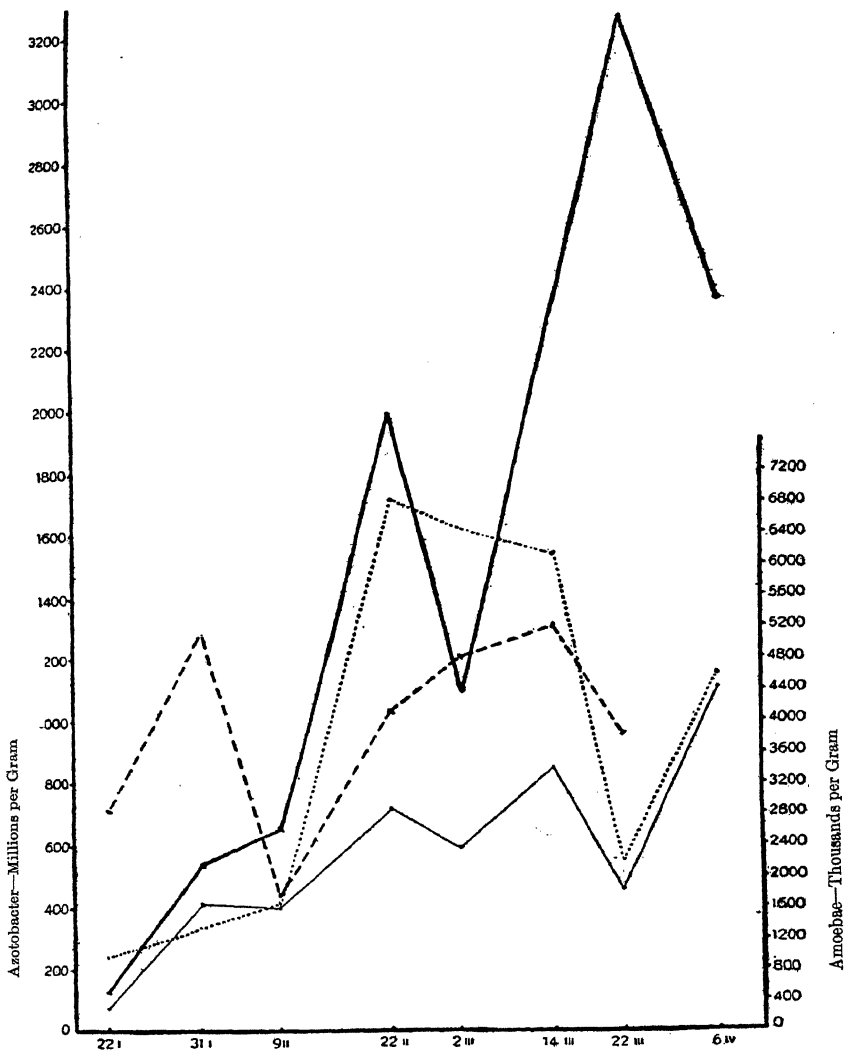


FIG. 10. Influence of amoebae upon the development of Azotobacter (from Fedorowa-Winogradowa). — Azotobacter in presence of amoebae; ..... amoeba in presence of Azotobacter; — Azotobacter alone; - - - amoeba alone.



result of autolysis. Impure cultures of *Azotobacter* contained considerable quantities of soluble nitrogen; this phenomenon was ascribed to the action of amoebae. A similar effect has been observed<sup>97</sup> in connection with the influence of protozoa on the liberation of ammonia by bacteria in peptone solutions: although the number of bacteria may be reduced, the process itself is favored.

These results show that although the protozoa are capable of reducing the number of bacteria in soil, due to their phagocytic action, there is very little evidence that their influence upon the activities of the microorganisms and upon soil processes in general is injurious. On the contrary, bacterial activities may even be favored, as indicated by an increase in the amount of ammonia liberated<sup>97</sup> or nitrogen fixed by *Azotobacter*. Excessive development of bacteria may become harmful to the growth of protozoa in artificial culture media, although it remains to be seen to what extent this may take place in the soil.

The fact that the protozoa destroy some soil bacteria need not indicate that they exert an injurious influence; this may even result in a decided benefit to soil biological processes. Decomposition of organic matter as well as other biological activities are resultants of the multiplication and growth of the bacterial cells. By destroying the excess of bacteria, the protozoa may stimulate further bacterial development and, therefore, further biological transformations in the soil. The protozoa themselves may become later a source of energy for bacteria. They may also play an important part in the soil by defending the plant against invasion by pathogenic bacteria.<sup>98</sup>

The protozoa probably take also a part in some definite soil processes, such as the decomposition of certain organic substances. Cleveland<sup>99</sup> found that protozoa living in the intestinal tract of termites feed on wood cellulose. When the protozoa are killed, the termites die on a wood diet, since they themselves cannot utilize cellulose, unless it has been previously decomposed by fungi. This symbiotic relationship exists for at least some protozoa. It is known that protozoa can readily assimilate soluble organic and inorganic constituents as found in manure and thus save the soluble substances from being leached out.<sup>100</sup>

<sup>97</sup> Meiklejohn, J. *Ann. Appl. Biol.*, 17: 614-637. 1930; Hill, T. L. *Jour. Bact.*, 1: 423-433. 1916.

<sup>98</sup> Hino, K. *Jour. Sci. Agr. Soc. Japan*, 239: 517-538. 1926.

<sup>99</sup> Cleveland, L. R. *Biol. Bul.*, 46: 177-225. 1924.

<sup>100</sup> Alexeiev, 1917 (p. 636). The relation of protozoa to reduction phenomena in the soil is discussed by von Wolzogen Kühr, C. A. H. *Arch. voor de Suikerind. nederland. Indie.*, No. 27, 1125-1182. 1917.

Koffman<sup>101</sup> concluded, as a result of extensive studies on the occurrence of protozoa and bacteria in the soil and the influence of the former on the metabolism of the latter that the microfauna of the soil exercises but an insignificant influence upon the microbiological processes in the soil. In this he confirmed similar conclusions reached by Waksman and Starkey in their study of the phenomenon of partial sterilization of soil, as shown later.

As to any direct relation of protozoa to growing plants, evidence has been submitted concerning the possibility that certain soil protozoa may cause direct injury to crops.<sup>102</sup>

<sup>101</sup> Koffman, M. Meddel. 391, Centralanst. forsoks. jordbruk. Bakt. Avd. 55, 1931.

<sup>102</sup> Sahasrabuddhue, D. L. and Bapat, G. M. Bombay Dept. Agr. Bul. 157, 1929.

## CHAPTER XIV

### THE NON-PROTOZOAN FAUNA OF THE SOIL

*Animal ecology as a whole and classification of soil forms.* In addition to protozoa, other groups of invertebrate animals inhabit the soil, namely rotifers, nematodes, rainworms, insects and others. The animals living in the soil can be generally divided into three groups:

1. Those that spend all their life in the soil, coming to the surface only occasionally or not at all. These include various worms and rotifers.
2. Those that spend only a part of their life cycle in the soil or on its surface, as in the case of various insects.
3. Those that find only their habitat in the soil, while they may spend a large part of their time on the surface of the soil. These include ants, termites and many insects.

The invertebrate animals influence directly or indirectly various soil processes and plant growth:

1. They cause a change in the physical condition of the soil, by modifying the mechanical structure of the soil, through their continued motion or by passing the soil through their bodies as in the case of earthworms.
2. They cause various chemical changes in the soil, either directly, in their digestive processes, or indirectly, by influencing the activities of the soil fungi and bacteria.
3. They bring about a more uniform distribution of various soil bacteria and other organisms.
4. They may devour other members of the soil flora and fauna, like algae, fungi and protozoa. In this way, the higher fauna also contributes to the complex system of numerous activities going on in the soil.
5. Damage may be done to crops by certain representatives of these groups, particularly by some of the nematodes, earthworms, insects, etc.

The soil, or terrestrial, fauna, outside of the protozoa, includes members of the following systematic groups:

- I. *Plathelminthes* or Flatworms, represented in the soil, in moist environments, by the (1) Turbellaria or flatworms and (2) Trematoda or flukes.
- II. *Nemathelminthes* or Roundworms, represented in the soil by the Nematoda or true roundworms.
- III. *Trochelminthes* or Trochalworms, represented in the soil by the Rotatoria or wheel animalcules.

- IV. *Coelhelminthes* (*Annelida*) or Segmented Worms are represented in the soil by the Oligochaeta, including the earthworms or Terricolae, and the Enchytraeids or Limicolae, and the Tardigrada.
- V. *Arthropoda* are represented in the soil by (1) Crustaceae, especially Copepoda and Isopoda; (2) Arachnida, including the mites, ticks and spiders; (3) Myriapoda; and (4) Insecta.
- VI. *Mollusca*, including the Gastropoda.
- VII. *Chordata*. The vertebrates are represented in the soil by the mice, moles, marmots, etc., but these are beyond our field of discussion.

*Methods of study.* For the investigation of the soil fauna, Morris<sup>1</sup> devised an apparatus, which consists of four iron plates, two 12 by 10 inches, one 12 by 9 and one 4 by 9 inches. Each plate has an iron bar fastened to it at the top, and each of the three larger plates has two projecting teeth at the bottom. The plates are driven into the ground down to the required depth to form a box 9 inches square, the smallest plate being on the side towards the outside of the plot. The plates enclose a cube of soil, with a side dimension of 9 inches, giving a total of 729 cubic inches. The soil is removed from the cube, in layers; the first sample contains only the upper inch of soil, the second and succeeding samples are taken at a depth of 2 inches at a time, giving in all five samples for each cube.

For making a census of the soil population, Cobb<sup>2</sup> devised soil sampling tubes, which are open cylinders of thin metal (tin or galvanized iron) with an internal diameter of 72.1 mm. The rim of one end is reinforced and the other sharpened. The area of the internal cross-section of the tube is one-millionth of an acre. The tubes may be of any length; for counting nematodes, 6 to 9 inch lengths are sufficient; below that depth, only few nematodes occur in the soil. Since the animal population is unevenly distributed in the soil, a number of samples are required, with a minimum of five. The various samples from one plot can be mixed and the census made. After the sampling tube is forced into the soil, enough earth is dug away to enable one to introduce a knife or saw-blade beneath the lower end of tube. The tube is then removed full of soil and capped at both ends. The samples of soil from one field are sifted and thoroughly mixed; wire sieves of  $\frac{1}{2}$  to  $\frac{1}{8}$

<sup>1</sup> Morris, H. Ann. App. Biol., 7: 141-155. 1921; 9: 282-305. 1922; Bull. Entom. Res., 13: 197. 1922.

<sup>2</sup> Cobb, N. A. Bur. Pl. Ind., U. S. Dept. Agr., Agr. Tech. Circ. 1. 1918. See also Baunacke, W. Arb. Biol. Reichsanst. Land. u. Forstw. 11: 185-288. 1922.

inch mesh may be used. Various methods of mixing<sup>129</sup> and sampling of the soil are described by Cobb.

An aliquot portion of soil is placed in an abundance of animal-free water, usually 10 to 20 times its volume. The soil is well suspended in the water by proper stirring with compressed air, carried on fast enough not to allow the particles to settle. The heavy particles are allowed to settle for about five seconds and the supernatant liquid is rapidly poured into another vessel. The residue is washed several times with clean water, so as to remove all adhering animals, the washings being added to the original liquid. The sand and gravel are discarded. The process may be repeated so as to remove another portion of the heavy inorganic material, being sure that it is free from animals. The liquid is then allowed to run through a series of superimposed sieves, ranging from 16 to 200 meshes per inch; the sieves, especially the finer ones, are agitated when the liquid is passed through them. The finer sieves may be made of millers bolting silk. The nematodes will all pass through the 16-mesh sieve; the residual particles should be washed so as to remove the animals. Beginning with the 20-mesh, the residual material should be examined carefully. To make sure that no animals remain in the liquid, the latter is passed several times (5 to 10) through the finest sieve. When a portion of the final liquid is examined and no nematodes are found, the liquid is finally discarded. The larvae of some animals, like those of Heterodera, are caught on the finest sieves. The animals are then washed away from the sieves (kept at a slightly inclined position) by a small amount of water. The washings with the animals are either mixed or kept in separate vessels.

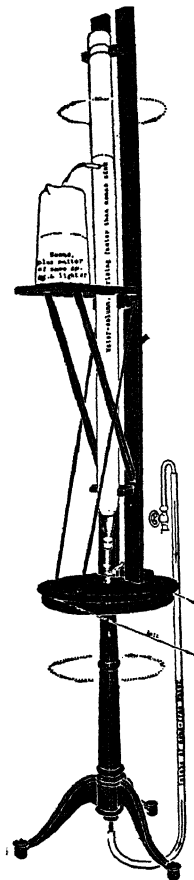


FIG. 11. Apparatus for the separation of nematodes from the soil (after Cobb).

The separation of the animals from the clay portion of the soil which is kept in suspension, is based upon the fact that they will settle quicker than the clay. Care should be taken that no animals are floating on the surface of the liquid. After the latter is allowed to stand for 30 minutes, it is poured off and replaced by clean water. The floating animals can be made to sink by adding some alcohol to the run-off material (so as to make 20 to 30 per cent alcohol), shaking well and adding water immediately (fig. 11). This process can be modified greatly depending on nature of soil.

The mixture of inorganic soil particles and organisms, lying in clear water, are then examined by using an ordinary dissecting microscope. Nematodes are fished out by slender, tapering, sharp needles free from

grease. A small portion of the debris is placed in a watch glass, in clear water, about one-eighth of an inch in depth. When a nematode is located, the point of the needle is brought under it and it is floated to the surface, lifted on the point of the needle and transferred to a watch glass containing a few drops of clean water. The final suspension may be well mixed and only an aliquot portion examined in a graduated watch glass. Examination should be made soon after washing is completed. Another procedure for counting the invertebrate animals in the soil, especially in forest soils, is described elsewhere.

Certain members of the animal, non-protozoan population can also be isolated by the use of agar media. Small worms can be isolated<sup>3</sup> by the following method:

Finely cut agar (3.5 grams) is soaked over night in tap water; excess of water is poured off and agar is dissolved in 200 cc. of tap water containing 1.3 grams of NaCl. The agar is neutralized and filtered; 1.6 grams of powdered brown sugar or malt sugar is then added, the agar is sterilized and distributed into sterile Petri dishes. The soil is inoculated, in the form of a fine layer, over the centre of the plate. The living worms will move away from the soil and, after 24 hours, they will be found on the clear agar about 0.5 cm. away. These worms, especially those bearing eggs, can be transferred to fresh agar plates, for the preparation of pure cultures.

#### FLATWORMS (PLATHELMINTHES)

The *Turbellaria* or free living flatworms are represented in the soil by various species of Rhabdocoelae, Allocoelae and land Planarians. Over thirty species of Rhabdocoelae were isolated from the soil.<sup>4</sup> Among the various genera found in the soil, it is sufficient to mention *Archivortex*, *Adenoplea*, *Acrochordonoposita*, *Geocentrophora*, *Prorhynchus* (*P. stagnalis*) and *Planaria*. The soil forms feed largely on diatoms, rotatorians, tardigrads, oligochaetes, upon one another and especially upon soil nematodes.

The *Trematoda* are represented in the soil by the larvae of different river flukes.

#### NEMATODA

Adult nematodes are usually cylindrical or spindle shaped, the posterior end being often acutely pointed or modified in form. They are

<sup>3</sup> Shaw, C. Centrbl. Bakt. II, 64: 41-45. 1925. Further information on the artificial cultivation of free-living nematodes is given by H. Metcalf. Trans. Amer. Micr. Soc., 24: 89-102. 1903; A. C. Chandler. Science, N. S., 60: Aug. 29. 1924.

<sup>4</sup> Reisinger, E. Turbellaria. Strudelwürmer. L. 6, T. 4, Schulze's Biologie der Tiere Deutschlands. Borntraeger. 1923.

transparent, non-segmented organisms, 20 to 100 times or more as long as wide; they are usually  $1.0\text{--}1.6 \times 0.3$  mm. in size, with a maximum length of 18 millimeters. When alive and active, they thrash about in pure liquid without making much progress. They do not change their length appreciably, being thus distinguished from earthworms and other elongated small organisms, which change their length while moving. Dead nematodes lie outstretched or in a slightly curved condition.

Nematodes are found in all soils under different conditions, largely in the upper 6–8 inches, although they are often abundant even at lower depths<sup>5</sup> (No. 144, Pl. XV). They can adjust themselves to various habitats. They are distributed by the wind, water, moving animals, various plant products, implements, etc. The eggs and larvae are sometimes very resistant to drying and other adverse conditions, and can survive for many years. Large numbers of parasitic, saprophytic, and free-living species inhabit the soil, making up a large numerical proportion of its population. The number of species alone reaches many thousands. Some of these are of wide distribution.

<sup>5</sup> Godfrey, G. H. Jour. Agr. Res., 29: 93–98. 1924.

## PLATE XV

### SOIL NEMATODES

144. The relative abundance of nematodes in each successive two inches of upper foot of soil; derived from a low-lying alluvial soil containing about 3,000,000,000 nematodes to the acre, most of which are in the upper 3 inches, around the plant roots (from Cobb).

145. Beneficial soil nematode, *Mononchus papillatus* Bastion: it feeds on other nematodes, showing remnants of several Tylenchuli (*j*, *t*) (from Cobb.)

146. Assymetrical nematode *Bunonema*, found in decomposing organic matter (from Cobb).

147. *Iota*, or scaly nematode, common in the soil; head and tail end of male and female (from Cobb).

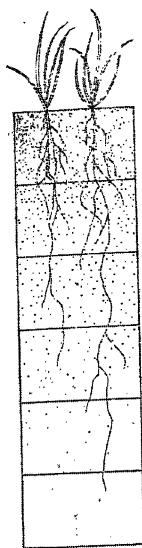
148. Male and female parasitic nematode, very simple in structure in comparison with free living nematodes (from Cobb).

149. *Tylenchus devastatrix* infecting onions and other bulbous plants (from Cobb).

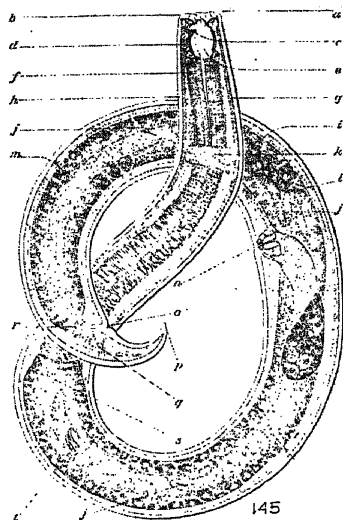
150. *Mononchus* attacking *Anguillula aceti* (from Steiner and Heinly).

151. Sketch of the head-end of *Mononchus* attacking a larval *Rhabditis* (from Steiner and Heinly).

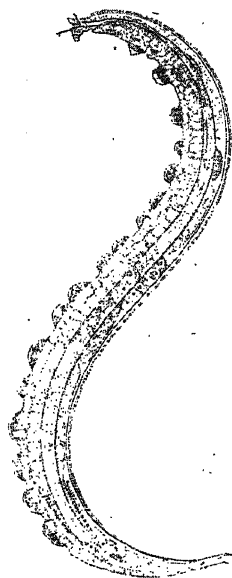
152. Schematic representation of the behavior of two different populations of *Tylenchus dipsaci*. The one population lived on Hyacinths, the other on Narcissus. Therefore, if left to choose, the first population will ignore the Narcissus, the second the Hyacinth, for each will attack only the host of its ancestors (Slogterem, after Steiner).



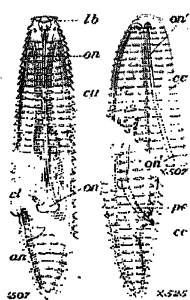
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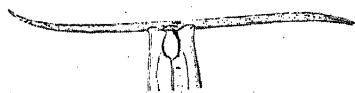
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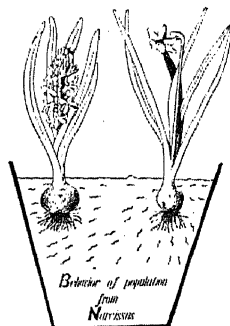
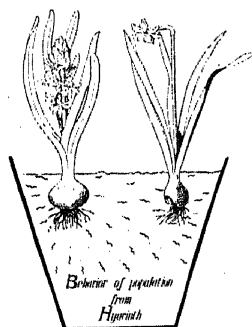
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148



152





The identification of soil nematodes may be carried out by using fixed material. Fleming's solution can be employed for this purpose. The organisms are placed in the solution from a few minutes to one or two hours. Should the cells become darkened, they can be bleached with  $H_2O_2$ . When the nematodes are dead and fixed, they are mounted, counted and identified, or are placed in a mixture of 5 per cent glycerol and 95 per cent water. After the water has evaporated, the animals remain in the glycerol. For careful identification and detailed study of morphology, the nematodes are placed in the middle of a glass slide in a small drop of water and covered with a cover glass. The edge of the cover glass can then be sealed to the slide by means of hot wax paraffin which contains a certain proportion of beeswax. The slides are now examined with the compound microscope, very high power lenses being necessary. De Man<sup>6</sup> was the first to make in 1884 a careful study of the soil nematodes. He divided the organisms into three groups: (1) omnivagous species not bound to any particular soil (*Dorylaimus obtusicaudatus*, *Monhystera filiformis*, etc.); (2) meadow and field soil nematodes (*Plectus cirratus*, etc.); (3) sand nematodes (*Mononchus parvus*, etc.).

Nematodes are generally found to be abundant in forest humus and in cultivated soils. They are parasitic on animals and plants, or are saprophytic and free living. Even the parasites may lead an independent existence in the soil at certain stages of their development. According to Steiner,<sup>7</sup> the nematodes are represented in Swiss soils by 139 known species, but there might be still many more. Cobb<sup>8</sup> found nematodes to occur in large numbers in every cultivated and uncultivated soil, including forms which are parasitic on plants or animals and those that are entirely saprophytic. Different soils may be found to contain large and varying numbers of nematodes as shown in the summary<sup>9</sup> on page 332.

One should keep in mind the fact that here only the free-living forms found in the upper 6 inches of soil were considered; it is known, however, that the nematodes can penetrate to much greater depths; when one remembers that the number of parasitic nemas must also be quite considerable, in some soils especially, one will recognize that the total number should be considerably greater than the above figures. Actu-

<sup>6</sup> De Man, 1922 (p. XIII).

<sup>7</sup> Steiner, G. Arch. Hydrobiol. Planktonk., 9: 259-276. 1913; Zool. Anz., 46: 336-368. 1916.

<sup>8</sup> Cobb, N. A. U. S. Dept. Agr. Yearbook, 1914, 457-490.

<sup>9</sup> Steiner, G. and Heinley, H. Jour. Wash. Acad. Sci., 12: 367-386. 1922.

ally as many as 15,914,272,000 nematodes were reported in the top 72 cm. of one acre of sugar beet soil in Utah.<sup>10</sup>

Morris<sup>11</sup> also found large numbers of nematodes in the soil, the greatest number occurring at a depth of two to three inches in manured soil and four to five in unmanured soil. Four to five times as many nematodes were reported to be present in manured as in unmanured soil. According to Micoletzky,<sup>12</sup> the soil and fresh water nematodes embrace 75 genera and 525 species. Half as many nematodes were found in the winter as in the summer. Steiner<sup>10</sup> states that some 800 species of nematodes belonging to about 100 genera have already been described from soils and that many more are still undiscovered. The abundance

CORN FIELD SOILS	MINIMUM NUMBER OF NEMATODES PER ACRE, TOP 6 INCHES (15.2 CM.)
Missouri.....	648,000,000
North Carolina.....	242,400,000
New Jersey.....	129,600,000
Rhode Island.....	610,800,000
New Hampshire.....	99,600,000
Minnesota.....	121,200,000
Vermont.....	580,000,000
Kansas.....	278,400,000

of these organisms in the soil is determined by moisture, aeration, soil structure, abundance of plant life and plant residues, penetration of plant roots and the presence of other microorganisms. The different nematodes vary in their nutrition:

1. Some, like *Rhabditis*, *Diplogaster*, *Cephalobus*, feed at least partly on decomposing organic matter which results from the activities of the bacteria, as well as upon the bacteria themselves, and also upon fungi and algae; this is also true of such forms as *Monhystera* and *Bunonema* (No. 146, Pl. XV).

2. Some feed on the tissues and fluids of healthy or injured plants, thus becoming injurious to higher plants; these include *Tylenchus*, *Heterodera*, *Aphelenchus* (Nos. 148-149, Pl. XV), some of which are truly parasitic and others are semi-parasitic (like *Hoplotaimus*) or facultative parasitic.

3. Some are parasitic on animals, especially invertebrate soil forms; certain nemas, parasitic on higher animals, including man, such as the hookworm, may pass part of their life cycle in the soil.

<sup>10</sup> Steiner, G. Proc. First. Int. Congr. Soil Sci., 3: 360-366. 1928.

<sup>11</sup> Morris, 1922 (p. 327).

<sup>12</sup> Micoletzky, H. Arch. Naturg., 87: 1-650. 1921; Zool. Anz., 64: 1-28. 1925; D. Kgl. Danske Vidensk. Selsk. Skr. Afd. 8 R., x, 2. København. 1925.

4. Some feed both on plants and animals, like *Dorylaimus*.
5. Some are parasitic on insects and can, therefore, be considered as playing a useful function.
6. Some are carnivorous and predatory, feeding on other soil organisms, such as molluscs, arachnids, myriapods, as well as nematodes. Here belong the genera *Ironus*, *Tripyla*, and especially the numerous species of *Mononchus*. *Mononchus papillatus*, for example, can feed readily upon *Heterodera radicicola*<sup>13</sup> (Nos. 150-151, Pl. XV).

The maximum number of nematodes was observed<sup>13</sup> in Tyrol soils in August, reaching 320 per 10 cc. of soil; the number then dropped rapidly and reached the lowest point in November, with 23 animals per 10 cc. It remained at a low level during the winter months and began to increase again in February. On the average, 120 nematodes were found per 10 cc. of soil throughout the year. The different species do not reach their maximum and minimum at the same time, depending on the moisture and the temperature resistance of the organism. The genera *Dorylaimus*, *Tylenchorhynchus*, *Mononchus* and *Hoplolaimus* are almost the only organisms found during the winter months.

The class Nematoda consists of numerous small forms which are usually free-living and non-parasitic. Among the free-living forms in the soil, the genus *Mononchus* composed of numerous species<sup>14</sup> is particularly abundant. Many of these species are cosmopolitan. The *Mononchs* occur in all kinds of arable soil, sometimes in hundreds of millions per acre. They feed on living microzoa, including other nematodes.<sup>14</sup>

Among the nematodes attacking the roots of various plants, causing the formation of galls, we find the sugar-beet nematode *Heterodera schachtii*, the root-knot nematode *Caconema radicicola*, the wheat nematode *Tylenchus tritici* and various others.<sup>15</sup> Some nematodes attack a great variety of plants. *C. radicicola*, for example, attacks about five hundred kinds of plants. This organism flourishes best in high, sandy soils, which are moist and warm. It attacks cotton, beans, celery, egg plants, potatoes, lettuce, peas, tomatoes, cowpeas, soybeans, nursery stock, weeds, ornamental plants and various field crops. Some plants, however, are not attacked. *Heterodera schachtii* attacks potatoes,

<sup>13</sup> Seidenschwartz, L. Arb. Zool. Inst. Univ. Innsbruck, 1: 37-71. 1923.

<sup>14</sup> Cobb, N. A. Soil Sci., 3: 431-486. 1917; De Man, J. G. M. Nijhoff. Hague. 1922; Wülker, G. Fadenwürmer. L. 11, T. 8, Schulze's Biologie der Tiere Deutschlands. Borntraeger. 1924; Shaw, H. B. Bur. Pl. Ind., U. S. Dept. Agr. Farm. Bul. 772. 1916. Baunacke, 1922 (p. 327).

<sup>15</sup> Marcinowski, K. Arl. Biol. Anst. Land. u. Forst., 7: 1-192. 1909.

sugar beets, etc., and may prove to be very injurious.<sup>16</sup> *Tylenchus dipsaci* attacks clover, alfalfa, and other crops, ornamental plants, etc.<sup>17</sup>

The nematodes are represented in the soil by a large number of genera.<sup>18</sup> It is sufficient only to enumerate some of the most common:

*Iota* (syn. *Hoplolaimus*) found in swamp and acid soils, on the roots of trees.  
*Tylenchus*, found in peat and moist soils, on plant roots; many parasitic species.

*Aphelenchus*, found in various soils, many species parasitic.

*Isonchus*, found on the roots of the cotton plant.

*Dorylaimus*, omnivagus, very abundant in the soil.

*Actinolaimus*, found in peat bogs and marshy soils.

*Ironus*, occurs to a limited extent in very moist soil.

*Mononchus*, predacious nematode, represented in the soil by many species, cosmopolitan.

*Diplogaster*, found in moist soils.

*Cyatholaimus*, found in moist soils.

*Plectus*, omnivagous, well distributed in the soil.

*Rhabditis*, cosmopolitan, some species microbivorous, well distributed in the soil.

*Rhabdolaimus*, found in moist soil.

*Cephalobus*, commonly found in the soil, can be grown on decomposing organic matter.

*Teratocephalus*, omnivagous, found in moist soil.

*Bastiana*, largely soil forms.

*Tripyla*, found in soils rich in undecomposed organic matter, not very abundant.

*Alaimus*, well distributed in the soil, especially in moist and forest soils.

*Prismatolaimus*, represented in the soil by some species.

*Monhystera*, omnivagous, found in moist soils, some feed on diatoms.

*Trilobus*, seldom found in moist soils, feeds on diatoms and rotatorians.

Various *Mermithidae* are also found in the soil.

A number of other genera, like *Bunonema*, *Tylopharynx*, *Archionchus*, *Eutylenchus*, etc., are found in the soil less abundantly.

<sup>16</sup> Zimmermann, H. Ztschr. Pflanzenkrank., 30: 139-145. 1920.

<sup>17</sup> Goodey, T. Jour. Agr. Sci., 12: 20-30. 1922; Ritzema-Bas, J. VI. Int. Congr. Inst. Agr., Paris, 2: 306-312. 1900. The cultivation of plant pathogenic nematodes is discussed by Byars, L. P. Phytopath., 4: 323-326. 1914; Hilgermann and Weissenberg, R. Centrbl. Bakt., I, Orig., 80: 467-472. 1918; Berliner, E. and Busch, K. Biol. Centrbl., 34: 349. 1914.

<sup>18</sup> A detailed classification of nematodes is given by Cobb, N. A. Fresh-water Biology by Ward and Whipple, 1918, p. 459-505; Micoletzky, 1921 (p. 332), and De Man, 1922 (p. XIII).

What is known of the nutrition of free-living soil nematodes has been reviewed in detail by Menzel.<sup>19</sup> He found that *Mononchus papillatus*, when brought together with *Tylenchus* sp., *Plectus auriculatus*, *Tripyla media* and *Anguillula aceti*, killed these forms either by swallowing them completely or by sucking out their contents. Steiner and Heinley grew the *Mononchus papillatus* in water containing some soil and placed in concave slides. It is important to use a small amount of soil free from excess of organic matter so as to prevent the rapid development of bacteria. The medium should be frequently renewed. *Heterodera*, *Rhabditis* and *Anguillula* were used for food. As many as 83 *Heterodera radicola* were killed in one day by one *Mononch*; during a life time of about 12 weeks, one animal killed 1332 nematodes. It is possible that this number may be much larger under natural conditions (No. 151, Pl. XV).

Steiner and Heinley, therefore, brought further weight to the suggestion of Cobb that the predatory *Mononchs* could be used to control the plant parasitic forms, when the latter are still free in the soil. However, we must keep in mind the fact that the mere introduction of an organism into the soil is not sufficient to insure its growth; the soil should be treated in such a manner as to favor the development of the beneficial organism and discourage that of the injurious forms.<sup>20</sup>

The rôle of nematodes in the soil may, therefore, consist of the following processes:

1. Consuming and destroying cultivated plants, often causing considerable damage.
2. Consuming soil bacteria and fungi.
3. Consuming soil protozoa.
4. Destroying other nematodes (predatory forms).
5. Distributing bacteria and fungi, including various plant and animal parasites, throughout the soil.
6. Puncturing various parts of plants and thus preparing an entrance for other parasites.
7. Taking an active part in the transformation of the soil organic matter.
8. Improving soil aeration.

#### ROTATORIA<sup>21</sup>

Rotatoria or Rotifera, commonly known as wheel animalcules, are minute, chiefly microscopic, animals. They are mostly characterized

<sup>19</sup> Menzel, R. Verhandl. Naturf. Gesell. Basel., 36: 153-188. 1920.

<sup>20</sup> Baunacke, 1922 (p. 327).

<sup>21</sup> Haring, H. K. U. S. Natl. Museum, Bul. 81, 1913; Haring, H. K. and Myers, F. J. Trans. Wiscon. Acad. Sci., 21: 415-549. 1924.

by the presence of a ciliated area, or corona, at or near the anterior end of the body, which serves both for locomotion and for bringing food to the mouth. Cilia are lacking on other parts of the body; in exceptional cases, they may be present at the posterior end. The corona may, in a few cases, be lacking. The body is usually somewhat elongated, with the corona at the anterior end and a tail-like appendage at the posterior end beyond the cloacal opening. The sexes are separate, the male being a minute, degenerate form, without an alimentary canal.

They are commonly found in swamps and marshes, as well as in moss and forest leaves. Francé found the following species in the soil:

*Rotifer tartigradus*  
*Rotifer vulgaris*  
*Philodina erythrophthalma*  
*Philodina aculeata*  
*Philodina vorax*  
*Adineta vaga*

*Callidina papillosa*  
*Callidina ehrenbergii*  
*Callidina multispinosa*  
*Habrotrocha angusticollis*  
*Diaschiza semiaperta*  
*Chaetonotus macrotrichus*

A number of other forms may occur, but they have not yet been made the subject of special study. Forty-nine species of Rotatoria were collected by Hauer<sup>22</sup> in sphagnum and other marshy places in Baden, including *Notommata copeus*, *Euchlanis lyra*, *Lecane amorpha*, *Mytilina bicarinata*, *M. trigona* and *Collotheca heptabrachiata*.

Attention must be called here to the fact that the various lists of animals found in the soil cover only a particular locality. The animal population of another locality, under different soil, climatic, and nutritive conditions, may be distinctly different in nature. Unfortunately our knowledge of animal life of the soil, except the protozoa, its distribution and economic importance is still so limited, that it is impossible at the present time to draw any broad generalizations.<sup>23</sup>

#### ANNELIDA

The annelids are represented in the soil by the earthworms (Oligochaeta-Terricolae), whose whole life cycle is passed in the soil, and by the white worms or Enchytraeids (Oligochaeta-Limicolae), which are usually abundant in moist soils, especially those rich in organic matter.

<sup>22</sup> Hauer, J. Schr. Ver. Gesell. Naturgesch. Baar Donaueschingen. H. 16: 252-272. 1926.

<sup>23</sup> P. de Beauchamp. Arch. Zool. exper. (4) 10: 1-410. 1909; Bull. Biol. France, 62: 52-125. 1928; Olofsson, O. Zool. Bidr. Upsala, 6: 185-646. 1918; Heinis, F. Arch. Hydrobiol. 5: 89-166. 1910; Lehmensick, R. Ztschr. wiss. Zool. 128: 37-113. 1926.

*Oligochaeta-Terricolae*.<sup>24</sup> The earthworms are characterized by their flexible segmented bodies, with four rows of bristles or setae. They have a well-defined body cavity and are hermaphroditic. The setae aid in locomotion. Various families are found in the soil.

The occurrence of earthworms in the soil has been of common knowledge since the work of Darwin and Hensen.<sup>25</sup> They are especially abundant in forest soils and soils rich in organic matter<sup>26</sup> and are almost absent in sandy soils. Heimbürger<sup>27</sup> suggested that a correlation exists between the degree of moisture of the soil and species of earthworms inhabiting it. The worms react definitely to atmospheric moisture but less sharply than to contact with moist substrate. They are commonly found in the upper 45 cm. of soil, but they may descend to a depth of 2 meters or more, if necessary.

To determine the numbers of earthworms in the soil, a certain volume of it is spread in a thin layer on a flat surface; when the soil begins to dry, the animals begin to move rapidly and can be counted. Moist soil may also be covered with a solution of sugar or powdered  $\text{KHSO}_4$ , which will bring the worms to the surface. Morris<sup>28</sup> found 1,010,101 earthworms per acre of manured soil and 457,912 per acre of unmanured soil. The greatest numbers occurred at a depth of two and three inches. The following species of earthworms were found in the soil by Francé: *Eisenia rosea*, *Lumbricus terrestris*, *Lumbricus rubellus*, *Allolobophora aporata*, *Helodrilus octaëdrus*. It was estimated that between 200 and 1,000 pounds of earthworms are present in an acre of soil. Thompson<sup>29</sup> found eighteen individuals in a nine inch cube of the upper three inches of a pasture soil.

Soil reaction has an influence upon the development of earthworms. They are most abundant at a pH of 7.0–7.8, and occur at a range of pH 5.6–8.3. Acid peat soils do not contain any earthworms.<sup>30</sup>

<sup>24</sup> Michaelsen, W. *Oligochaeta*. Das Tierreich. No. 10, 1900; Stephenson, J. The oligochaeta. Clarendon Press, Oxford. 1930.

<sup>25</sup> Darwin, Ch. Vegetable mould and earthworms. London. J. Murray. 1881; Hensen, V. Ztschr. wiss. Zool. 28. 1877; Landw. Jahrb., 11: 661–698. 1882.

<sup>26</sup> Remelé, E., Schellhorn, and Krause, M. Ztschr. Forst. u. Jagdwes., 31: 575–606. 1899.

<sup>27</sup> Heimbürger, H. V. Ecology, 5: 276–282. 1924.

<sup>28</sup> Morris, H. M. Rothamsted Station Rept. for 1918–1920, p. 20.

<sup>29</sup> Thompson, M. Ann. Appl. Biol., 11: 349–394. 1924; see also Bassalik, K. Ztschr. Gärungsphysiol., 2. 1913.

<sup>30</sup> Arrhenius, O. Ecology, 2: 255–262. 1921; Gleisberg, W. Ztschr. angew. Bot., 4: 234. 1922; Kahsnitz, H. G. Boruss. Arch., 1: 315. 1922. Allee, W. C., Torvik, M. M., Lahr, J. P. and Hollister, P. L. Physiol. Zool., 3: 164–200. 1930.



The worms feed not only upon plant residues, but also on other soil organisms. Francé found various algae, fungus mycelium, protozoa and yeasts in the excreta of earthworms. The animals pass earth through their bodies, grinding it in the gizzard into fine particles and decomposing some of the organic matter which may be present. The earth is then passed out of the body and deposited as castings at the surface of the burrows. The soil is thus well mixed with the organic matter and brought from the lower layers to the surface. According to Darwin, ten tons of earth for each acre of land may be passed through the bodies of the earthworms every year. This mechanical action of the worms upon the structure of the soil is of great importance. Wollny<sup>31</sup> considered that the worms take part in the decomposition of nitrogenous compounds in the soil. Soils containing earthworms and upon which grass was growing was found to contain more ammonia, nitrate and total nitrogen than soils without worms and grass; this is probably due more to the grass than to the worms.<sup>32</sup>

Stöckli,<sup>33</sup> who made recently a detailed study of the rôle of earthworms in soil processes, found that the worms excreted in a year 2.02 kgm. of material for 1 sq. meter of forest soil and 8.13 kgm. for pasture soil. In the course of 30 years, this will amount to a layer of 5.4 and 19.8 cm. thickness respectively. The activities of the worms are at a maximum in the spring and fall of year, due to the optimum moisture and temperature conditions. The worms are found even in very acid soils, although the use of ammonium sulfate as a fertilizer is commonly recommended for the elimination of the worms.<sup>34</sup> They take in a large amount of undecomposed or partly decomposed dead plant residues, digest them and mix them thoroughly with the inorganic soil particles, producing thereby important chemical changes in the organic matter thus digested. One of the results of this digestion is an increase in the so-called "humus" or alkali soluble organic matter.

It was suggested<sup>35</sup> that earthworms increase plant growth by increasing the surface of soil due to excreta, thus affecting the water-holding

<sup>31</sup> Wollny, E. *Die Zersetzung der organischen Stoffe*. 1897, p. 39.

<sup>32</sup> Blanck, E. and Giesecke, F. *Ztschr. Pflanzenernähr. Düng.*, **3B**: 198-210. 1924.

<sup>33</sup> Stöckli, A. *Landw. Jahrb. Schweiz.*, **42**: 1-121. 1928.

<sup>34</sup> Walton, W. R. *U. S. Dept. Agr. Farm. Bull.* 1569. 1928.

<sup>35</sup> Kohswitz, H. G. *Bot. Archiv.*, **1**: 315-331. 1922; Aichberger, R. V. *Die Kleinwelt*. **6**, 1914; Heymons, R. *Ztschr. Pflanzenernähr. Düng.*, **2A**: 97-129. 1923.

capacity of the soil and movement of water. According to Russell,<sup>36</sup> earthworms do not appear to have any marked effect on the production of plant food; their chief work is to act as cultivators, loosening and mulching the soil, facilitating aeration and drainage by their burrows. Stöckli found, however, that earthworms exert a decidedly favorable effect upon various important groups of soil bacteria, including the cellulose-decomposing, nitrogen-fixing, including both *Cl. pastorianum* and *Azotobacter*, etc. The action of the aerobic bacteria is particularly favored due to the improvement of soil aeration. The addition of earthworms to partially sterilized soil had a markedly favorable effect; they also removed the injury caused to crops by addition of plant residues and favored an increase in nutrient substances in an available form.<sup>37</sup>

Earthworms may prove objectionable when present in great abundance in lawns, especially in putting greens of golf links. This is especially marked on lawns receiving applications of organic manures. Lead arsenate is usually recommended for their control; this is applied at the rate of 1 pound to 1000 sq. feet of surface. A detailed discussion of the occurrence and function of earthworms in forest soils is given elsewhere (p. 673).

*Oligochaeta-Limicolae* or *Enchytraeidae*. This family is characterized by their whitish appearance and presence of more than two straight setae in some of the bundles. Moist soils, especially those rich in organic matter, will contain large numbers of these organisms. Thompson observed as many as 86 forms in a 9-inch cube of the upper 3 inches of pasture soil, including several species of *Fredericia* and *Enchytraeus* (*E. albidus*). Francé found species of *Enchytraeus*, *Fredericia* and *Anachaeta* in the soil. According to Jegen,<sup>38</sup> these worms are capable of neutralizing the injurious effect of certain nematodes in the soil; he also claimed that they play a rôle in the formation of humus in the upper soil layers and considered their activities as even of greater importance in soil than that of the earthworms. They may also become injurious to plant growth since they feed on germinating seed of beets and upon other young plants; however, their food consists largely of decomposing plant tissues. They are very sensitive to drying and to lack of oxygen.

Their relative abundance is indicated by the following numbers found in one square meter of different soils, at different seasons of the year. They are practically absent in heavy clay soils.

<sup>36</sup> Russell, E. J. Jour. Agr. Sci., 3: 346. 1910.

<sup>37</sup> Arkhangelski, M. Nauch. Agron. Zhur., 6: 849-862. 1929.

<sup>38</sup> Jegen, G. Landw. Jahrb. Schweiz, 34: 55-71. 1920.

Numbers of *Enchytraeidae* in a square meter of soil

SOIL TYPE	LOAM	SANDY SOIL	HUMUS SOIL
Spring.....	60- 100	6,900- 9,000	30,000- 70,000
Summer (dry).....	28- 75	2,600- 4,900	11,800- 16,000
Summer (moist).....	70- 300	6,500- 8,500	28,000- 50,000
Autumn.....	100- 450	7,000-11,000	60,000-150,000
Winter.....	800-1,600	6,800- 9,400	50,000-120,000

*Tardigrada*. The tardigrads are a group of Annelida, although they are often wrongly classified with the Arachnida and Arthropoda.<sup>39</sup> The organs of locomotion are unarticulated, with more or less retractible parapodia. The body is 1 to 10 mm. long, cylindrical, often almost worm-like. Some are without eyes, some have compound eyes in the form of black or red spots. The sexes are separate. During a period of dryness, they hibernate. Hibernation may last for years without injury to the organism. The tardigrads can withstand considerable heat and cold. Under unfavorable conditions, they encyst; regeneration of the organs follows this stage. Various species are found in the soil. Francé found this group represented by the genera *Macrobiotus* and *Milnesium*.

#### ARTHROPODA

*Crustacea*. The crustaceans are represented in the soil by the *Copepoda* (family *Harpacticidae*) and the *Isopoda*,<sup>40</sup> or higher crustaceans. The Harpacticidae do not have the cephalothorax and abdomen distinctly separated, so that the body is worm-like. Francé found in the soil one species of *Moraria* and six species of *Canthocamptus*. 33,700 to 80,000 Isopods were recorded per acre of soil by Morris. The presence of crustaceans in the soil was also reported by Thompson.

#### ARACHNIDA

The Arachnids are represented in the soil by the mites and ticks (Acarina<sup>41</sup>), which are chiefly carnivorous, free-living or parasitic, and

<sup>39</sup> Richters, F. Handwörterbuch der Naturwissenschaften., 9. 1913; see Marcus, E. Zool. Jahrb. Abt. allgem. Zool. Physiol. Tiere, 44: 323-370. 1928; Brown's Klassen und Ordnungen des Tierreichs, 5: Abt. 4, B. 3. Leipzig. 1929.

<sup>40</sup> Van Douwe, C. and Neresheiner, E. Copepoda. Die Süßwasserfauna Deutschlands. H. 11, 1909; Richardson, H. U. S. Nat. Mus. Bul. 54. 1905.

<sup>41</sup> Wolcott, R. H. Trans. Amer. Micr. Soc., 26: 161-243. 1905; Dogiel, V. Rev. Zool. Russe, 4. 1924.

by the carnivorous spiders (Areinida). Morris found the Acarina represented in the soil by the Amystidae, Tarsonemidae and Tyroglyphidae. The presence of various members of the Trombidiidae and Oribatidae in the soil was also reported by Thompson. *Porrhomma*, *Robertus*, *Oedothorax*, *Linyphia*, and others are the genera of Areinida found in the soil by Morris. The greatest numbers of Acarina were found in the upper one inch of soil, the total number being 531,986 per acre of manured soil and 215,488 per acre of unmanured soil. The mites take an active part in the decomposition of the organic residues in the soil.

#### MYRIAPODA

Among the myriapods present in the soil, are the millepedes (*Diplopoda*) which attack various crops, the centipedes (*Chilopoda*), which are carnivorous, and the *Symphyla*.

Many of the millepedes, as the Colobognatha, are slow moving, unable to burrow in the soil or withstand surface exposure. They are found only in the organic matter layer of the soil and are controlled by a continuous availability of moisture. Most of these animals are found in the tropics. Very few genera, such as *Polyzonium* and *Hypozonium*, have been found in the temperate regions, quite distinct from the tropical fauna.<sup>42</sup>

The following species of myriapods were found in soils of temperate regions.<sup>43</sup> *Glomeris hexasticha*, *G. frausalpina*, *Polydesmus* sp., *Craspedosoma rawlinsii*, *C. canestrinii*, *Chordeuma nodulosum*, *Ch. silvestre*, *Julus nigrofuscus*, *C. verhoeffi*, *Schizophyllum sabulosum* and *Lithobius forficatus*. Morris found 1,781,143 myriapods in the upper nine inches of an acre of manured soil and 878,787 in the corresponding unmanured soil. They were distributed more or less uniformly with depth. The Diplopoda were represented by species of *Brachydesmus*, *Cylindroiulus*, *Blaniulus* and *Archiboreoiulus*; the Chilopoda by *Lithobius*, *Geophilis* and *Geophilomorph*. *Symphyla* were also found in both soils.

The myriapods feed upon the living and dead organic substances in soil. Many attack earthworms and other animals considerably larger than they in size. Millepedes have often been blamed for injury to crops brought about by other animals largely the wireworms.

#### INSECTA

The term "soil insect" comprises all insects which, at one time or another in the course of development from the egg to the imago, spend

<sup>42</sup> Cook, O. F. and Loomis, H. F. Proc. U. S. Nat. Mus. 72: 1-26. 1928.

<sup>43</sup> Diem, K. Jahrb. St. Gall. Naturw. Gesell. Vereinsjahr. 1901-1902.

some stage or stages of their life-cycles either on or beneath the surface of the soil. A great many species of insects, including most orders, are associated with the soil in one or more stages of their development. As a matter of fact, it has been stated that as many as 95 per cent of all insect species invade the soil at some stage of their development. Millions of insects are found in every acre of arable land.

On the basis of their feeding habits, the soil insects can be divided into six groups:<sup>44</sup>

1. Those feeding on subterranean parts of plants, as the larvae of *Melolontha*, *Agriotes* and *Tipula*.

2. Those living saprophytically in the soil, as *Collembolla* and larvae of *Diptera* and *Coleoptera*.

3. Those living on other members of the soil fauna, or predaceous species, as the *Carabidae* and many larvae of *Diptera*.

4. Parasitic species, as the *Hymenoptera* and the *Tachinidae*, which pass their larval stages on or within the bodies of other organisms.

5. Insects which find their habitat in the soil, without seeking a food supply there, as in the case of ants.

6. Insects which only undergo pupation in the soil, as in the case of the *Lepidoptera*.

A survey of the insect fauna in cultivated and pastoral lands revealed the fact that the distribution and numbers of the soil fauna are more stable on grass than on arable land.<sup>45</sup> This is due to the fact that grassland bears a vegetative covering all the time, which offers food for the fauna. In grass land, hibernation can proceed normally. Cultivation of land brings the fauna to the surface exposing it to harsh climatic conditions and to bird attack. As vegetative growth increases, there is a corresponding increase in the fauna in both arable and grass land. Conditions in winter and early spring are detrimental to the soil fauna.

The fauna of arable land consists of species which have passed the winter in the soil and those which have migrated or are introduced during the growing season. There is no characteristic fauna in cultivated land. Buckle isolated from the soil one species of *Collembolla*, 35 species of *Coleoptera*, 6 *Diptera*, 2 *Hymenoptera*, 4 *Chilopoda* and *Diplopoda*.

A detailed study of the insect fauna of the soil has also been made

<sup>44</sup> Cameron, A. E. Science Progress, No. 77: 92-108. 1925; Imms, A. D. In book of Sir John Russell et al. The microorganisms of the soil. Longmans, Green & Co. 1923.

<sup>45</sup> Buckle, P. Ann. Appl. Biol., 8: 135-145. 1921.

by Cameron, Morris and Thompson.<sup>46</sup> Morris found 2,475,000 insects in the upper nine inches of an acre of unmanured plot and 7,727,000 in a manured plot. The dominant groups in both plots were the Collembola and Formicidae; the Chironomidae larvae and Trichocera larvae were much more abundant in the manured plot. The Collembola were represented by 14 species: the Thysanura by 3 species, the Orthoptera and Thysanoptera by one each; the Hemiptera by 4; the Lepidoptera by unidentified larvae; the Coleoptera by 30 species; the Diptera by 7 species and various unidentified larvae; the Hymenoptera by 10 species. The greatest majority of the organisms were found in the upper three inches of soil. The wireworms attain a maximum at a depth of 5 to 7 inches. Manuring increases the total number of soil organisms about 200 per cent, but has no appreciable influence on the number of wireworms (fig. 12).

Morris also found 3,586,088 insects in an acre of permanent pasture, the numbers of the different orders being Collembola—566,680, Rhynchota—15,140, Thysanoptera—43,258, Lepidoptera—15,140, Coleoptera—744,038, Diptera—2,193,180, Hymenoptera—8652. Among the injurious insects, the following were found per acre: Agriotes—114,643 larvae and 8652 adults, *Triphaena pronuba*—4326 larvae and pupae, *Tipula oleracea* and *T. paludosa* 19,466 larvae. The family most represented was the Bibionidae, the species of this family making up 32.4 per cent of the total number of soil insects; Mycetophilidae was represented by 16.7 per cent and Staphylinidae by 12.2 per cent. The Coleoptera were represented by 29 species. According to Thompson, the orders Collembola and Acarina determine the trend of the total fauna curve, since they are the dominant groups. They persist throughout the year, while other groups like the Nematoda and Oligochaeta may be entirely missing for varying lengths of time. Cultivated land contains a considerably smaller population than pasture or grass land; the maximum population was found to occur in the winter months, due to sufficient moisture in the soil. Peat soils also contain a definite fauna of insects, as represented by the Collembola.<sup>47</sup> These organisms

<sup>46</sup> Cameron, A. E. Jour. Econ. Biol., 8: No. 13. 1913; Trans. Roy. Soc. Edin., 52: pt. 1, No. 2. 1917; Morris, H. M. Ann. Appl. Biol., 14: 442-463. 1927; Thompson, 1924 (p. 337); Holthaus, K. Ztschr. wiss. Insektenbiol., 6: 1-4, 44-57. 1910; Adams, C. C. Ill. State Lab. Nat. Hist. Bul. 11: Art. 2. 1915; Shelford, V. E. Geogr. Soc. Chicago, Bul. No. 5; Univ. Chicago Press. 1913; Vestal, A. G. Ill. State Lab. Nat. Hist. Bul. 10: Art. 1. 1913.

<sup>47</sup> Handschin, E. Beitr. Kunde Estlands., 10: 167-176. 1924.

are also abundant in forest litter and are frequently highly specialized. The dead human bodies in the soil were found to carry a definite fauna of Collembola.<sup>47a</sup>

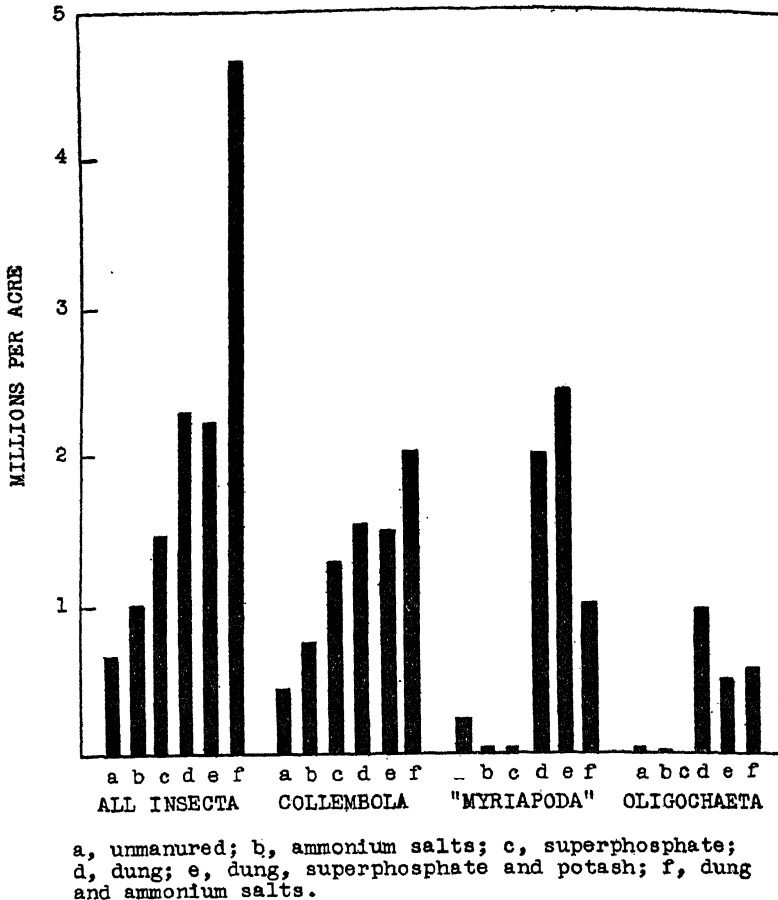


FIG. 12. Numbers of the more important groups of insects in various soil plots (from Morris).

M'Atee<sup>48</sup> reported the presence of 1,216,880 animals belonging to Insecta, Arachnida and other Arthropoda, Annelida and Gastropoda in

<sup>47a</sup> Folsom, J. W. *Psyche* (Cambridge), 9. 1902.

<sup>48</sup> M'Atee, W. L. *Science*, N. S., 26: 447-449. 1907.

an acre of forest soil. An abundant insect fauna has also been demonstrated in soils of arid regions, as in Central Asia.<sup>49</sup> This fauna was distributed with depth as follows: 0-1 cm. horizon—300,000 individuals per hectar; 1-5 cm.—1,750,000; 5-10 cm.—3,000,000; 10-20 cm.—1,500,000; 20-40 cm.—1,000,000; 40-60 cm.—1,500,000. The most abundant groups were various Coleoptera, including the Carabidae, Scarabaeidae, Curculionidae, Histeridae, Staphylinidae, etc., as well as various Hymenoptera, especially the Formicidae. The Hemiptera were most abundant in 1-5 cm. level, the Diptera and Formicidae in the 0-20 cm. and the Coleoptera throughout the 0-70 cm. depth. Ants were found to prefer loam soils and avoid sandy soils. The abundance of termites in soil is pointed out later.

A detailed study of the subterranean aphids has been made by Cutright.<sup>50</sup>

#### MOLLUSCA

The molluscs possess a soft body encased in a hard shell consisting either of one part (Gastropoda snails) or two parts (Lamellibranchiata). The soil molluscs include the snails and the slugs, which frequently leave the soil which they inhabit for feeding purposes in the presence of sufficient moisture. Most of these forms usually consume vegetable matter, while some (*Testacella*) are carnivorous. The genera *Carychium* and *Helix* were reported in the soil by Francé.<sup>51</sup>

*Influence of environmental conditions on the invertebrate fauna of the soil.* Larvae of soil insects are very sensitive to evaporation of moisture, especially at 20°C. or over.<sup>52</sup> They do not occur in dry exposed soils, but rather in moist soils where the humidity is not far below saturation and the temperature seldom goes above 20° to 23°C. Below 8°C., most soil insects become inactive. Hibernating soil insects possess a great capacity of resisting freezing temperatures. They migrate vertically, according to season, especially in cold climates.<sup>53</sup> The invertebrate fauna of the soil is generally much more abundant in heavy than in light sandy soils because of the moisture conditions.<sup>54</sup>

<sup>49</sup> Brodsky, A. and K. Bull. Inst. Pedol. Geobot. Univ. Asie Central., 3: 151-166. 1927.

<sup>50</sup> Cutright, C. R. Ohio Agr. Exp. Sta. Bul. 387: 175-238. 1925.

<sup>51</sup> The influence of soil reaction upon snail development is discussed by Atkins, W. R. G. and Lebour, M. V. Nature, 11: 83. 1923.

<sup>52</sup> Hamilton, C. C. Biol. Bull. 32: 159-182. 1917.

<sup>53</sup> Griddle, N. Agr. Gaz. Canada, 5: No. 5. 1918.

<sup>54</sup> Ramann, E. Int. Mitt. Bodenk., 1: 138-164. 1911.



According to Morris the depth to which insects penetrate into the soil is due to depth of food, aeration, moisture and soil temperature; insects are found, therefore, at greater depths in arable soil than in pasture land.

The addition of organic matter to the soil increases the moisture holding capacity of the soil and offers food material for many species. Different soil insects respond differently to varying degrees of humidity, some selecting light, sandy soils and others living only in soils saturated with water. The optimum soil habitat is determined by the ratio or balance between the amount of available oxygen and the amount of carbon dioxide which can be endured without injury. Loam soils have a more abundant insect fauna than clay and sandy soils.<sup>55</sup>

According to Edwards,<sup>56</sup> the vertical distribution and seasonal behavior of the soil invertebrate population depends upon soil type, as well as the moisture, aeration and temperature conditions of the soil. The greatest number of organisms occurs in pasture areas in the surface inch layer, but some species occur at a greater depth. When a soil is cultivated there is an increase in certain types (Symphyla, Diplopoda, some Collembola) and a reduction in other types (Oligochaeta, Acarina, Coleoptera, Diptera, certain Collembola). Light soils are more favorable to the multiplication of the soil pupating species of thrips than clay soils, while cultivation of soil increases infestation by these insects.<sup>57</sup>

The great majority of free-living soil nematodes are "world wide" or are found everywhere when conditions are favorable for their development. To what extent this is true of the other members of the invertebrate fauna of the soil still remains to be investigated.

*Economic importance of the invertebrate fauna of the soil.* The importance of the invertebrate fauna in the soil consists in (1) the mechanical effect upon the soil, as discussed above; (2) the relation of the fauna to the transformation of organic substances in the soil; (3) the relation of the invertebrates, especially of the insects and certain nematodes, to the growth of higher plants. The influence of the fauna upon the bacteria, fungi, actinomyces and algae of the soil is still a matter of speculation. There is no doubt that the invertebrate animals feed to some extent upon the soil microflora and thus influence its activities. The earlier idea of Pasteur that the distribution of anthrax bacteria in the soil is brought about by worms inhabiting the soil may hold true also for the soil microflora as a whole.

<sup>55</sup> Adams, 1915 (p. 343); Hesse, R. Tiergeographie auf ökologischer Grundlage. Jena. 1924; McColloch, J. W. Jour. Amer. Soc. Agr., 18: 143-159. 1926.

<sup>56</sup> Edwards, E. E. Ann. Appl. Biol., 16: 299-323. 1929.

<sup>57</sup> McGill, E. J. Ann. Appl. Biol., 17: 150-161. 1930.

McColloch<sup>58</sup> suggested that there is a reciprocal relation between the soil and its insect population: these utilize the soil for shelter, protection, as an avenue for travel, and find there their food, moisture and atmosphere. The soil is benefited, on the other hand, by being well mixed, by the mechanical separation of particles, improvement of aeration and drainage, and addition of organic matter. The mechanical effect may prove injurious as a result of the fact that the soil may become porous leading to an increase in evaporation and plant injury.

According to Morris, the nitrogen content of the invertebrate fauna varies from 4.88 per cent for the Myriapoda to 11.18 per cent for the Collembola, making a total nitrogen content of the fauna of an acre of manured ground 16.2 pounds and 7.5 pounds for unmanured ground. More than half of this nitrogen was in the bodies of the earthworms. The action of worms and insects in assisting the breaking down of vegetable matter, with the formation of amorphous "humus," was considered<sup>59</sup> of importance.

The soil insects are frequently classified<sup>59</sup> into three groups: beneficial, noxious and innocuous. The first include such forms as species of Ichneumonidae and Braconidae, which are parasitic on cutworms, predatory Carabidae and scavenging Scarabaeidae. The noxious insects are found among the Elateridae, Noctuidae, some Scarabaeidae, Curculionidae and Tipulidae. The innocuous forms are those which find in the soil a temporary retreat for pupation; even injurious species, like the potato-beetle, may be harmless during the soil phases.

The larvae of *Oryctes nasicornis* dig into the soil, swallowing particles of woody plant material mixed with soil. The organic matter is digested especially the cellulose. The flagellates inhabiting the digestive tract of the larvae feed upon the bacteria and play no part in the digestion of cellulose. The bacteria inhabiting the tract decompose the cellulose and when they are in their turn digested by the enzymes of the host, they become available nutrients to the latter.<sup>60</sup> The rôle of bacteria in the decomposition of cellulose by insects has also been pointed out previously (p. 189).

Among the most important insects whose larvae may become of great economic importance in the soil in injuring field crops, are the wire-

<sup>58</sup> Kostytschew, P. Russian tschernoziems. Petrograd. 1886, p. 165-191. Ann. Sci. Agr., 2: 1887; Ann. Agron., 17: 17-38. 1891.

<sup>59</sup> Cameron, 1925 (p. 342).

<sup>60</sup> Wiedemann, J. F. Ztschr. morph. Ökol. Tiere, 19: 228-258. 1930.

worms, white grubs and cutworms.<sup>61</sup> The parents of the wireworms deposit their eggs chiefly in grass land. The small wireworms which hatch out feed on the roots of various plants or seeds before they are sprouted, especially when the crop is planted after sod. This continues until the insects are fully mature, which requires three to five years. The damage is usually most severe in spring. An abundance of wireworms in sandy soil frequently makes it necessary to abandon or "rest" the land.

The parents of the white grubs are the June bugs which also lay their eggs principally in sod land during June. The eggs hatch in about two weeks. The grubs feed on any plants available and go down seven to fourteen inches below the surface when cold weather approaches. With the coming of warm weather the following spring, they come up again towards the surface where they feed on plants throughout the season.<sup>62</sup> This is repeated twice; the common cycle being three years. This white grub may become a limiting factor in continuous wheat production in certain sections; the infestation usually increasing with each generation, so that it becomes necessary to rotate with a cultivated crop. The green June beetle and muck beetle also prefer soils receiving heavy applications of animal manures.

The cutworms hatch in September or October and become very active during the following spring. They cut off young plants near the surface of the soil and lap up the exuding sap. This is usually done at night.

Crop rotation, use of artificial fertilizers, fall plowing,<sup>63</sup> the use of poisoned and other baits, soil fumigants (CS<sub>2</sub>) and insecticides (Paris green), as well as the direct mechanical protection of plants, are among the remedies suggested for these three pests. A detailed discussion of the methods of soil treatment for control of soil-born injurious insects is given by Friederichs.<sup>64</sup>

Insects may also inhibit the activities of certain specific soil organisms,

<sup>61</sup> Headlee, T. J. N. J. Agr. Exp. Sta. Cir. 26; Anderson, G. M. S. C. Agr. Exp. Sta. Bul. 204, 1920; Thomas, W. A. S. C. Agr. Exp. Sta. Bul. 155, 1911; Hawkins, J. H. Maine Agr. Exp. Sta. Bul., 343, 1928.

<sup>62</sup> McColloch, J. W. and Hayes, W. P. Ecology, 4: 29-36. 1923; Osborn, H. Proc. 39th Ann. Meet. Soc. Prom. Agr. Sci., 7-18. 1919.

<sup>63</sup> Hyslop, J. A. U. S. Dept. Agr. Bul. 156. 1915; Hunter, W. D. U. S. Dept. Agr. Yearbook, 1911: 201-210. 1912; Treherne, R. C. Entom. Branch, Dept. Agr., Canada, Pamphl. 33; Davis, J. J. Soil Sci., 10: 61-76. 1920.

<sup>64</sup> Friederichs, K. Die Grundfragen und Gesetzmäßigkeiten der land und forstwirtschaftlichen Zoologie. 2 vols. P. Parey, Berlin. 1930.

such as legume bacteria, by feeding on the nodules. This was pointed<sup>65</sup> out for the beet leaf larva *Cerotoma trifurcata*, and for the mealy bugs *Pseudococcus maratinus*.<sup>66</sup> As many as fifteen bugs were seen on one soybean nodule.

It is sufficient to call attention to the rôle of termites (*Hodotermes*) in certain soils, to obtain an idea as to the probable importance of the animal population in soil processes. Termites live in tropical and subtropical countries, not only in wooden structures, in which they cause active decomposition of organic matter of living and dead plants, but also in the soil itself. The underground termites are found in great abundance in dry countries and in deserts. They form a great abundance of nests, which are connected by a net of underground passages. These termite nests made the soil very porous, with the result that there is a great increase in the amount of water required for saturation of the soil. The activities of the termites in the soil result in increases in the concentration of salts. These increases may be from 0.06 to 1.3 per cent. The termites, as a result of the numerous passages formed in the soil, appreciably improve soil drainage.<sup>67</sup>

Francé suggests that there exists an association in soil (*Edaphon*) consisting of bacteria, algae, fungi, diatoms, protozoa, rotatoria, nematodes, worms, myriapods and insects. The bacteria and fungi liberate nitrogen for the algae; all three forms serve as food for rhizopods and together with these they serve as food for rotatoria and nematodes; the latter are eaten by amoebae, myriapodes, insects, etc.; these are, in turn, decomposed by the fungi and bacteria.

<sup>65</sup> McConnell, W. R. Jour. Econ. Entom., 8: 261-267. 1915; 8: 551. 1915; Leonard, L. T. and Turner, C. F. Jour. Amer. Soc. Agr., 10: 256-261. 1918.

<sup>66</sup> Leonard, L. T. Science, N. S., 57: 671-672. 1923. See also Folsom, J. W. Ill. Agr. Exp. Sta. Bul., 134. 1909.

<sup>67</sup> Escherich, K. Die Termiten. Leipzig. 1909; Dimo, N. A. In Soil and Bot. Geogr. Investig. of the basins of Amu-Daria and Sir-Daria. Moskau, 2: 1-38. 1916; see also S. F. Light. Cir. 314, California Agr. Exp. Sta. 1929.



PART C

CHEMICAL ACTIVITIES OF MICROORGANISMS

*“Für alle Lebewesen ist ein nie fehlendes Kennzeichen der Energiestrom. Meist bezeichnet man den hier stattfindenden Vorgang mit dem Namen Stoffwechsel. Dieses Wort trifft aber nicht die Hauptsache.”—*WI. OSTWALD.



## CHAPTER XV

### GENERAL PRINCIPLES OF MICROBIAL METABOLISM

*Metabolism as a whole.* To be able to understand the chemical processes taking place in the soil as a result of the activities of microorganisms and to learn how to control these processes, so as to produce conditions which make a soil productive and thus benefit the growth of higher plants, we must understand the metabolism of the various groups of soil microorganisms. The biological changes produced in the soil fall under the class of chemical reactions. However, the biologist is dealing with dynamic phenomena, while the chemist considers chiefly static processes. This is the reason why a chemical analysis of a soil is far from sufficient to give us information as to productivity of the soil, or the rapidity with which the nutrients necessary for the growth of higher plants become available. Not only the chemical changes must be considered as such, but also the course or rate of change. This can be done and the information, subject to a host of variable factors, can be properly interpreted only when the metabolism of the organisms concerned is taken into consideration.

The metabolism of some of the more common and important groups of soil microorganisms can be considered under the transformation of carbon, of nitrogen and of mineral compounds. From the point of view of soil productivity, various microorganisms may be considered to play important rôles in certain specific processes, depending on the nature of the organism and nature of the medium. The various transformations in the soil dovetail and, for a proper understanding of the resulting phenomena, metabolism should always be considered as a whole.

The carbon compounds are used by the heterotrophic microorganisms as sources of energy and as sources of carbon for structural purposes, or for the building up of the microbial cell. In both cases the carbon is required in the form of complex organic compounds, such as carbohydrates, hydrocarbons, fats, fatty acids, proteins and their split products, including amino acids and acid amides. Some organisms prefer one group of compounds and some another, while some can utilize a variety of substances as sources of carbon. A great many of the known soil bacteria are more or less selective in their action (when grown, of course,



upon artificial culture media); many soil fungi and actinomyces and a number of bacteria can derive their carbon, both for energy and structural purposes, from a great variety of substances. *Bact. pyocyaneum*, for example, can obtain its carbon not only from carbohydrates, but also from lactic and acetic acids, glycerol, ethyl and methyl alcohols, and other substances.<sup>1</sup> Some bacteria are highly specific, being able to use only one compound or a closely related group of compounds.

The autotrophic bacteria need no complex carbon compounds as sources of energy or for structural purposes. They can derive their carbon for the synthesis of their protoplasm from the carbon dioxide of the atmosphere or in solution. The facultative autotrophic bacteria can obtain their carbon either from CO<sub>2</sub> or from organic compounds. There is some evidence, however, that growth of heterotrophic organisms is also favorably affected by the presence of CO<sub>2</sub>, as in the case of *Bac. subtilis* and *Bact. vulgare*, which could not grow when both oxygen and carbon dioxide were removed.<sup>2</sup> *Asp. niger* spores germinate only very poorly in an atmosphere free from CO<sub>2</sub>. It has been suggested that the CO<sub>2</sub> imparts a proper swelling state to the protoplasts.<sup>3</sup> The presence of carbon dioxide is essential not only for the growth of aerobic organisms but also for the development of the anaerobic bacteria.

In view of the fact that the microbial cells contain between 3 and 15 per cent of nitrogen, large quantities of this element have to be assimilated, particularly by organisms producing an extensive growth. Nitrogen is obtained from proteins and their degradation products or simple inorganic nitrogenous compounds, including the ammonium salts of organic and inorganic acids and nitrates. Some organisms, especially certain heterotrophic bacteria, prefer and many even require complex proteins, albumoses or peptones as a source of nitrogen (and energy), while other microorganisms, especially fungi and autotrophic bacteria, will thrive just as well and sometimes even better upon simple compounds of nitrogen. *Bact. pyocyaneum* can obtain its nitrogen from amino compounds, amides, nitrates and nitrites, but these substances must be changed, either by hydrolysis or by reduction, to ammonia before they are assimilated. The nitrogen-fixing microorganisms, capa-

<sup>1</sup> Supniewski, J. *Biochem. Ztschr.*, **154**: 90-97, 98-103. 1924.

<sup>2</sup> Rockwell, G. E. *Jour. Infec. Dis.*, **32**: 98-104. 1923; **35**: No. 6. 1924; **38**: 92-100. 1926; Valley G., and Rettger, L. F. *Jour. Bact.*, **14**: 101-138. 1927; *Quart. Rev. Biol.*, **3**: 209-274. 1928; Novy, F. G. et al. *Jour. Inf. Dis.*, **36**: 109. 1925.

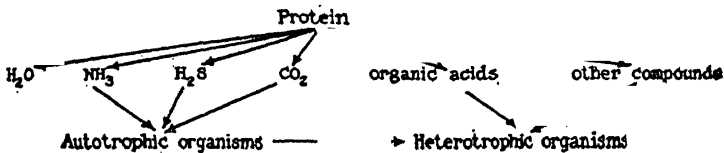
<sup>3</sup> Rippel, A. and Bortels, H. *Biochem. Ztschr.*, **184**: 237-244. 1927.

ble of utilizing free nitrogen gas, in the absence of available compounds of this element, stand as a group by themselves.

The minerals, chiefly phosphates and potassium salts, but also iron, magnesium, sulfur, calcium and traces of other elements, are utilized by all microorganisms either in the form of simple inorganic compounds or are obtained from complex organic substances in the process of their decomposition. The minerals may often be obtained from insoluble inorganic materials, especially if the organism produces acids which tend to make them soluble.

In the utilization of nutrients by heterotrophic microorganisms, two general stages are observed. (1) The dissimilation or decomposition stage, in which organic matter is broken down by the agencies of hydrolysis, oxidation and reduction. (2) Assimilation stage, or synthesis, whereby the cells of microorganisms are built up out of the substances previously broken down.

The metabolism of autotrophic bacteria consists only of the synthesizing stage so far as organic substances are concerned. The autotrophic microorganisms utilize for their synthesis the products of dissimilation of the heterotrophic organisms, such as the various minerals, nitrogen compounds and even as energy sources such as ammonia, hydrogen sulfide, etc. The heterotrophic microorganisms utilize for their dissimilation stage the products of assimilation of the autotrophic forms, namely the complex organic substances synthesized by these cells.



Only the autotrophic organisms actually produce work, in the true thermodynamic sense, as shown later, while the heterotrophic forms may simply build up the new protoplasm out of the constituents of the medium. This explains the considerably greater assimilation and the more extensive protoplasm produced by the heterotrophic organisms for the same amount of energy available.

*Chemical reactions in the microbial cells.* The microbial cell may be considered as an osmotic system. The absorption and liberation of substances by microorganisms lead to a series of chemical reactions

necessary for the continuation of life and characteristic of the living cell. Most of these reactions are carried on in the cell by the agency of organic catalysts or enzymes, which may also be secreted outside of the cell; this allows certain chemical reactions to take place outside of the cell.

The chemical reactions depend on the presence of specific substances or substrates, on the chemical and physical condition in which these substances are present, on temperature, reaction, etc. The activities of the microorganisms will result in a change both in nature of the substrate as well as in the condition of the medium in which they work. Celluloses and proteins, substances of high molecular weight and low osmotic pressure, will be changed by processes of hydrolysis, to sugars and organic acids or to peptides and amino acids, substances of low molecular weight and high osmotic pressure. On the other hand, the absorption of soluble nitrogen salts and minerals and their synthesis into microbial protoplasm will bring about a reverse condition. The ionic exchange in the living cell, as the absorption of the base in the case of ammonium salts or absorption of the acid in the case of nitrates, will tend to leave the medium more acid or more alkaline respectively. The formation of organic acids, such as gluconic, citric, oxalic and fumaric by fungi, lactic, butyric, formic, propionic, valerianic and acetic by bacteria, and inorganic acids, such as carbonic, nitrous, nitric and sulfuric, will also lead to a change in the hydrogen-ion concentration of the medium. These acids will combine with the insoluble and soluble bases forming new salts. Some of these, such as the salts of organic acids, may be used as sources of carbon, liberating the bases which will combine with the carbon dioxide of the atmosphere to form carbonates, and again change the reaction of the medium from acid to alkaline. Others, like the nitrates and sulfates, may be again assimilated by microorganisms and higher plants. These may be washed from the soil in the drainage waters, they may be absorbed by the soil colloids, or they may form simple or complex salts with the various inorganic or organic soil constituents.

All of these reactions bring about constant changes in the osmotic concentration and the reaction of the medium. This is further accentuated by the formation of electrolytes from non-electrolytes (ammonia and nitrates from proteins and amino acids, phosphates and sulfates from complex protoplasm) and vice versa. It is important, therefore, to gain knowledge of the osmotic concentration of the soil solution, as determined by the lowering of the freezing point, change

in conductivity,<sup>4</sup> or other convenient method; also of the hydrogen-ion concentration, as determined by the electrometric or colorimetric method,<sup>5</sup> and of the buffer content of the soil, as determined by the curves which show the relation between addition of acid and alkali and change in reaction.<sup>6</sup>

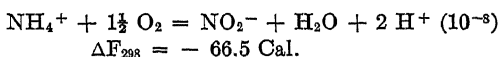
*Energy transformation by microorganisms.* The autotrophic bacteria obtain their energy chemosynthetically, or by the oxidation of chemical elements or simple inorganic compounds; the chlorophyll-bearing algae utilize the photosynthetic energy of the sun; the heterotrophic microorganisms, comprising the great majority of bacteria, all fungi, protozoa and other invertebrate animals obtain their energy from carbohydrates, proteins and other complex carbon compounds, either by direct oxidation (aerobic) or by processes of oxidation-reduction (anaerobic). In the course of the life activities of the cell, growth and reproduction, a part of the energy is used for the synthesis of complex organic compounds of the cell and a part is transformed into heat and dissipated into space. The heterotrophic microorganisms utilize the complex carbon compounds as (1) sources of energy, (2) for structural purposes, (3) for the building up of reserve substances; a part of the nutrients may be left in a non-modified or in a modified form as a waste product of metabolism.

The transformation of energy by microorganisms is usually calculated either on the basis of heat of reaction or of free energy,  $\Delta F_{298}$  always referring to the free energy decrease or the maximum amount of useful work obtainable from the process at 25°C. or 298° absolute temperature.<sup>7</sup>

The process of oxidation of ammonium salt by *Nitrosomonas* can thus be presented:



or as



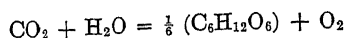
<sup>4</sup> Bouyoucos, G. J. and McCool, M. M. Mich. Agr. Exp. Sta., Tech. Bul. 24, 1915; 27, 1916; 31, 1916; 37, 1917; 43, 1918; Hoagland, D. R. Jour. Agr. Res., 12: 369. 1918; Hibbard, R. P. and Chapman, C. W. Mich. Agr. Exp. Sta., Tech. Bul. 23, 1915.

<sup>5</sup> Clark, W. M. 1928 (p. XVIII).

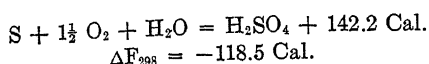
<sup>6</sup> The amphoteric character of bacterial cells is discussed by Stearn, E. and Wand, A. E. Jour. Bact., 10: 13-23. 1925; Univ. Missouri Studies, 3: No. 2. 1928; Reiss, P. Le pH intérieur cellulaire. Paris. 1926.

<sup>7</sup> Baas-Becking and Parks. Physiol. Rev. 7: 85-106. 1927. Buchanan and Fulmer, 1928 (p. XIII); Burk, D. Jour. Phys. Chem., 35: 432-455. 1931; Wilson, P. W. and Peterson, W. H. Chem. Rev. 8: 427-480. 1931.

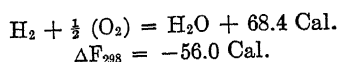
It is believed that for the synthesis of its protoplasm, the organism obtains its carbon from the  $\text{CO}_2$  of the atmosphere according to the following reaction:



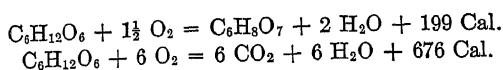
The oxidation of sulfur by *Thiobacillus thiooxidans*:



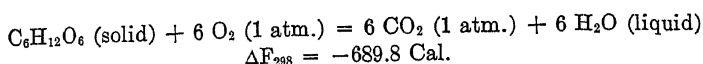
The oxidation of hydrogen by bacteria:



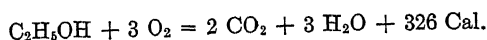
Aerobic utilization of energy by fungi:<sup>8</sup>



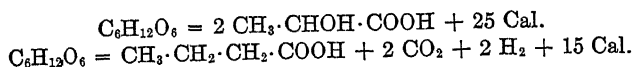
or



Oxidation of alcohol by bacteria.



Anaerobic utilization of energy:<sup>9</sup>



The amount of energy liberated in oxidation processes is considerably greater than that made available by the anaerobic fermentations. Much larger quantities of substrate have to be decomposed in the latter processes than in the oxidation by free oxygen, in order to liberate the same amount of heat and allow an equal growth of the cells to take place. The growth of microorganisms upon a substrate is parallel to the energy value of the nutrient, provided other conditions are the same. This is

<sup>8</sup> Kruse, 1910 (p. XIII); Terroine, E. F. et al. Compt. Rend. Acad. Sci., **174**: 1435. 1922; **175**: 228; **177**: 900-902; **178**: 809, 1488. 1924; Bull. Soc. Chim. Biol. **7**: 351-379. 1925; **8**: 584-603. 1926.

<sup>9</sup> Meyerhof, O. Biochem. Ztschr., **162**: 43-86. 1925; Quastel, H. et al. Biochem. Jour., **19**: 304-317, 660-666. 1925.

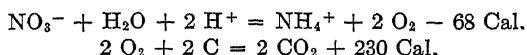
illustrated in table 23. The nature of the waste products of metabolism is influenced by the composition of the medium, type of organism and conditions of growth. The products formed as a result of growth of a

TABLE 23  
*Influence of energy source upon the growth of fungi (Kruse)*

SOURCE OF ENERGY	ENERGY EQUIVALENT OF 1.5 GRAM OF MATERIAL	FUNGUS MYCELIUM SYNTHESIZED
	<i>calories</i>	<i>gram</i>
Tartaric acid.....	2.6	0.155
Citric acid.....	3.7	0.240
Glucose.....	5.6	0.278
Glycerol.....	6.5	0.475
Peptone.....	6.8	0.162
Olive oil.....	14.0	0.810

bacterium upon a nitrogenous organic compound are shown in table 24.<sup>10</sup>

When the oxygen required for oxidation is obtained by the reduction of nitrates, a part of the energy is thereby consumed in the reduction process:



or

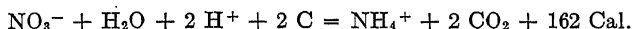


TABLE 24  
*Growth of Bact. pyocyaneum on a 0.5 per cent asparagine solution*

	CARBON	NITROGEN
	<i>per cent</i>	<i>per cent</i>
Assimilated by the bacteria.....	13.8	4.66
Given off as CO <sub>2</sub> or NH <sub>3</sub> .....	72.5	91.0
Non-volatile products.....	13.5	4.04

Since the organic matter added to the soil has a calorific value of 4.6 to 5.0 Cal. per gram, the addition of 3 tons of dry, ash-free organic matter, in the form of stable manure or green manure, per acre of soil, introduces 13,000,000 Cal. available for the growth of microorganisms. This organic matter begins to undergo rapid decomposition by micro-

<sup>10</sup> Arnaud, A. and Charrin, A. Comp. Rend. Acad. Sci. 112: 755-758, 1157-1160. 1891.

organisms, whereby a part of the energy is stored away in the microbial cells, a part is dispersed into space in the form of heat, while a part may remain for a long time in the form of resistant original or modified plant constituents, or as intermediary substances of decomposition.

*Growth, life and death of microorganisms.* The numbers of bacteria and other microorganisms in soil vary greatly not only from day to day, but even within brief periods of time, as a result of the changes in the environmental conditions. The bacteria themselves may reproduce within twenty to thirty minutes. Growth of an organism can be continued indefinitely, when repeatedly transferred upon fresh media. In the same medium, however, there is at first a decided increase in the

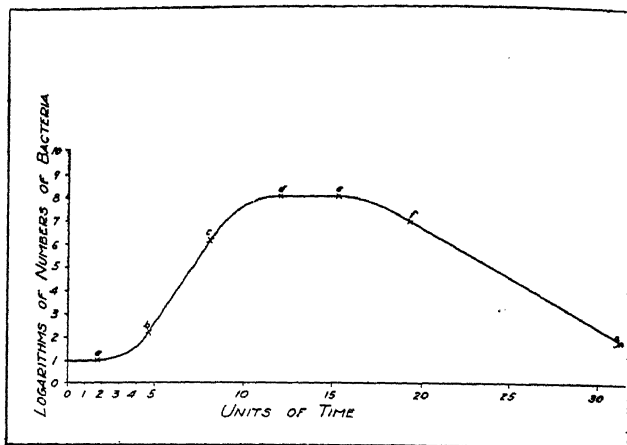


FIG. 13. Rate of increase in numbers of a bacterium (from Buchanan).

numbers and activities of a microorganism, reaching a maximum sooner or later; this maximum may be followed by a rapid decrease or may continue for a certain period of time. The death rate of the organism is usually much slower than the growth rate, as shown in fig. 13.<sup>11</sup>

The rate of growth and the death rate of different organisms vary. The maximum of growth reached by microorganisms in a limited amount of medium is due to the exhaustion of one or more nutrients or to the formation of injurious by-products, such as acids, alkalies or some toxic substances.<sup>12</sup> While the older cells die off, the younger continue

<sup>11</sup> Buchanan, R. E. Jour. Inf. Dis., **23**: 109-125. 1918.

<sup>12</sup> Chambers, W. H. Ann. Mo. Bot. Gard., **7**: 249-289. 1920; Meyer, R. Ztschr. Pflanzen. Düng. A, **8**: 121-163. 1927.

to grow and use the nutrients made available by the decomposition (autolysis) of the older cells.

A microbial culture thus passes through a period of youth, full development and old age. These stages can be expressed by the autocatalytic curve.<sup>13</sup> A difference exists in the physiological activities and in the physico-chemical condition of young and mature cells.<sup>14</sup> A medium, in which growth of a certain organism has ceased, as a result of accumulation of an injurious substance, may be treated so as to neutralize the accumulated acid or alkali, or the limiting nutrient may be

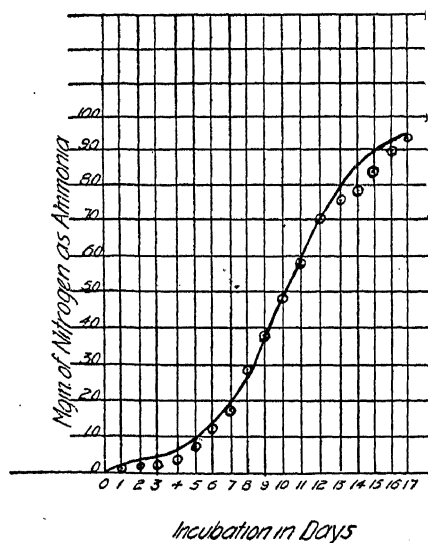


FIG. 14. Rate of ammonia formation from peptone by *Asp. niger* (after Waksman).

added, or finally the toxic substance may be destroyed by means of heat.<sup>15</sup> The organism will begin to grow again and pass through another cycle of activities. Limitations of growth and formation of certain substances (ammonia, nitrate) may thus be due either to reactions of autocatalysis, or perhaps more likely to the development of a certain balance between the reproduction of the cells and the accumulation of

<sup>13</sup> Robertson, T. B. Arch. Entw. Mech., **37**: 497-508. 1913; Univ. Cal. Publ. Physiology, **4**: 211-228. 1915.

<sup>14</sup> Sherman, J. M. and Albus, W. B. Jour. Bact., **8**: 127-138. 1923.

<sup>15</sup> Rahn, O. Centrbl. Bakt. II, **16**: 417-429, 609-617. 1906.



injurious products of metabolism.<sup>16</sup> Miyake,<sup>17</sup> using the results of Lipman and associates on ammonia accumulation and of Warington on nitrate accumulation in the soil, calculated that these processes are autocatalytic chemical reactions. The maximum rate occurs when the total amount of transformation is half completed. The amount of ammonia or nitrate increases according to the formula:

$$\frac{dx}{dt} = Kx (A - x)$$

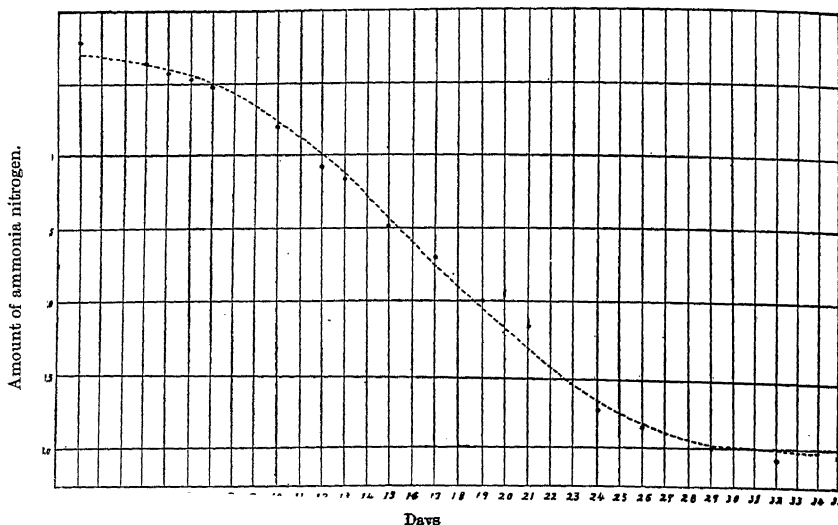


FIG. 15. Rate of oxidation of ammonia to nitrate, as shown by disappearance of ammonia in soil (after Miyake and Soma).

in which  $A$  is the initial quantity of material subject to transformation,  $x$  is the amount transformed in time  $t$ , and  $K$  is a constant. Similar results have been obtained for ammonia formation<sup>18</sup> from peptone by *A. niger* and for  $\text{CO}_2$  evolution by fungi in the decomposition of carbohydrates.

The different microorganisms are not growing in the soil in pure

<sup>16</sup> Meller, R. Centrbl. Bakt., I, 64: 1-32. 1925.

<sup>17</sup> Miyake, K. Soil Sci., 2: 481-492. 1916. Jour. Biochem. Tokyo, 1: 123-130. 1922; Lipman, J. G., Blair, A. W., Owen, I. L. and McLean, H. C. N. J. Agr. Exp. Sta. Bul. 247, 1912.

<sup>18</sup> Waksman, S. A. Jour. Bact. 3: 475-492. 1918.

culture. In the presence of a great number of various other organisms, stimulative and injurious substances may be formed constantly.<sup>19</sup> It has even been claimed that soil microorganisms produce substances (nucleic acids) which stimulate the growth of cultivated plants.<sup>20</sup>

*Chemical composition of the microbial cell.* The presence of a certain number of organisms in a given soil and at a given time is not due to a mere accident, but because a definite amount of energy, nitrogen and minerals are made available for their growth and reproduction in a given period of time, and as a result of definite environmental conditions. A change in the energy supply and in the environment produce a change not only in the numbers of microorganisms, but also in the nature of the soil population. Different numbers and different kinds of microorganisms are found in a given soil, at various periods, because varying quantities of nutrients are made available. When a soil is air-dried and again moistened, there is an increase in the numbers and activities of microorganisms, because the process of drying results in rendering a greater amount of the inorganic and organic matter available. When a fresh amount of undecomposed organic matter is added to the soil, a rapid increase in numbers of microorganisms takes place because a large amount of the energy added is readily available.

The cell substance of microorganisms can be divided into (1) the cell wall, (2) microbial cytoplasm, and (3) nuclear material; many organisms also contain (4) capsular material which may be quite distinct in composition.

The cell wall is rich in chitin  $[\text{CH}_2\cdot\text{OH}\cdot(\text{COOH})_3\cdot\text{CHNH}_2\cdot\text{CHO}]_n$ . The cytoplasm of bacteria contains various metachromatic granules, chromatin-like bodies described as volutin, glycogen, fat particles, and various waxy or sulfur granules. The capsular material consists of mucin, glycoprotein and carbohydrates. Very little is known concerning the nuclear material of the microbial cell. The presence of cellulose and hemicelluloses in the bacterial cell wall has been claimed by some and denied by others.<sup>21</sup> By means of microchirurgical operations, it has been shown that bacterial cells are provided with a membrane of enormous elasticity. The membrane is in a condition of a resistant gel, while the inner contents of the cell form a sensitive colloid in a sol con-

<sup>19</sup> Pringsheim, E. G. *Centrbl. Bakt.* II, 51: 72-85. 1920.

<sup>20</sup> Mockeridge, F. A. *Ann. Bot.*, 38: 723-734. 1924.

<sup>21</sup> Zellner, J. *Chemie der höheren Pilze*. Leipzig. 1907; Buchanan and Fulmer, 1928 (p. XIII); Vaughan, V. C. *Chem. Rev.* 4: 167-188. 1927.

dition, but may be changed to a gel. No nucleus could be demonstrated.<sup>22</sup>

Different bacteria differ radically in their chemical composition, both in the relative proportions of fats and carbohydrates and in the character of the constituent proteins. *B. tuberculosis*, for example, contains an appreciable amount of water soluble protein of the albumin type, while *B. lactis aerogenes* does not. The determination of nitrogen distribution in the alkali-soluble protein by the Van Slyke method also shows that each bacterium is apparently characterized by its own specific protein, that of *B. tuberculosis* containing a much higher percentage of arginine and a lower percentage of lysine and amide nitrogen than the protein of *B. lactis aerogenes*. The latter organism was found to contain 11.05–11.38 per cent nitrogen, 1.34–1.62 per cent phosphorus and 3.41–5.25 per cent ash (the  $P_2O_5$  content of the ash is 65.5–69.0 per cent). The bacteria are first extracted with ether and with cold water (10 per cent of the nitrogen and 24 per cent of the phosphorus are water soluble, mostly dializable), followed by extraction with 5.0 per cent NaCl, then with 0.5 per cent NaOH in the cold.<sup>23</sup>

To illustrate further the nature of the investigations dealing with the composition of bacterial cells, especially soil organisms, the following citations will suffice. Cramer<sup>24</sup> found that the average water content of bacteria grown on agar media was 87.71 per cent. The dry material consisted of 50.47 to 51.81 per cent carbon, 6.59 to 7.49 per cent hydrogen, 12.32 to 13.46 per cent nitrogen and 7.79 to 10.36 per cent ash. On media rich in carbohydrate the nitrogen content was often less than 10 per cent. The ether-alcohol extract varied from 9.06 to 24.0 per cent, depending on the organisms and the composition of the medium. According to Nicolle and Alilaire,<sup>25</sup> bacteria contain, on the basis of dry weight of cells, 8.3 to 10.8 per cent of nitrogen. The moisture content of the cells ranged from 73.4 to 85.5 per cent; acetone extracted 6.3 to 15.6 per cent of the dry material and chloroform 1.5 to 11.8 per cent. The chloroform extract contained 0.2 to 2.5 per cent phosphorus.

<sup>22</sup> Wamoscher, L. Ztschr. Hyg., 111: 422–460. 1930.

<sup>23</sup> See Hetler, D. M. Jour. Biol. Chem. 72: 573–585. 1927; T. B. Johnson, Amer. Rev. Tuberc., 14: 164. 1926; Jour. Biol. Chem., 54: 721, 731. 1922; 70: 449. 1926; Colloid Symposium Ann. 7: 223–232. 1929; Parisi, E. and Masetti-Zannini, C. Staz. sper. Agrar. ital., 59: 207–228. 1926; Goadby, K. Proc. Roy. Soc. B., 102: 137–142. 1927; Stiehr, G. Centrbl. Bakt. II, 71: 265–268. 1927.

<sup>24</sup> Cramer, E. Arch. Hyg., 16: 151–195. 1893.

<sup>25</sup> Nicolle, M. and Alilaire, E. Ann. Inst. Past., 23: 547–557. 1909.

*Rhizobium meliloti*<sup>26</sup> was found to contain 0.6–1.2 per cent ether-soluble and 10.2–21.7 per cent chloroform-soluble material; only a small part of the fat was in the form of simple lipids; the total carbon varied from 52.8 to 54.6 per cent, and the total nitrogen from 4.38 to 4.91 per cent of the dry material.

Algae grown on synthetic nutrient agar media, with  $\text{Ca}(\text{NO}_3)_2$  as the only source of nitrogen, were reported<sup>27</sup> to contain 7.4 per cent nitrogen, on an ash-free basis. About 25 per cent of this nitrogen could be extracted with water and consisted of proteins, amino acids and peptides. The nitrogen content of fungi varies between 2.3 and 6.6 per cent, depending on the nature of substrate.<sup>28</sup> Soon after the spores germinate and active cell formation takes place, the nitrogen content of the fungi may be much higher. A study of the abundance of nitrogen in the cells of bacteria, fungi and actinomyces, grown on media containing an amino

TABLE 25  
*Nitrogen content of some typical soil organisms*

NAME OF ORGANISM	1 PER CENT GLUTAMIC ACID	1 PER CENT GLUTAMIC ACID + 2 PER CENT GLUCOSE
	<i>per cent</i>	<i>per cent</i>
<i>Bact. fluorescens</i> .....	10.0	10.0
<i>Act. viridochromogenus</i> .....	8.0	8.0
<i>Zygorhynchus mölleri</i> .....	6.8	4.6
<i>Trichoderma koningi</i> .....	6.9	5.3

acid as the only source of carbon and nitrogen, shows<sup>29</sup> that both the nature of the organism and the presence of non-nitrogenous substances are of great importance in this connection.

The nitrogen content of some fungi may be as low as 1 per cent,<sup>30</sup> in the absence of available nitrogen and in the presence of an excess of energy compounds. It may be as high as 8–11 per cent, in the absence of sufficient energy and in the presence of an excess of nitrogen. The

<sup>26</sup> Hopkins, E. W., Peterson, W. H. and Fred, E. B. Jour. Biol. Chem., 85: 21–28. 1929.

<sup>27</sup> Fred, E. B. and Peterson, W. H. Bot. Gaz., 79: 324–328. 1925.

<sup>28</sup> Mazé, P. Ann. Inst. Past., 16: 346–378. 1902; Nikolsky, M. Centrbl. Bakt. II, 12: 554–559, 656–675. 1904.

<sup>29</sup> Waksman, S. A. and Lomanitz, S. Jour. Agr. Res., 30: 263–281. 1924.

<sup>30</sup> Gerlach, M. and Vogel, I. Centrbl. Bakt. II, 10: 636–643. 1903; Czapek, F. Beitr. Chem. Physiol. Path. 1: 538, 2: 557, 3: 47. 1902.

wide variation is due to the adaptation of the organisms to the composition of the media. The nature of the organism is of course of great importance in determining its chemical composition.<sup>31</sup> The concentration of nutrients has also a marked influence upon the composition of fungus mycelium (table 27).<sup>32</sup>

TABLE 26  
*Influence of age of culture upon the nitrogen content of a fungus (Nikolsky)*

AGE, DAYS	TOTAL DRY MYCELIUM	NITROGEN PRESENT	NITROGEN
	<i>gram</i>	<i>gram</i>	<i>per cent</i>
4	0.46	0.075	16.2
6	0.51	0.043	7.1
8	1.12	0.062	5.6
10	1.67	0.104	6.2
12	1.72	0.090	5.2
14	1.83	0.068	3.7

The nitrogen content of fungi is usually much lower than that of bacteria, showing that the nitrogen-free substances predominate in their mycelium. Zopf<sup>33</sup> found that the dry weight of higher fungi consists of 11-51 per cent protein, 2.15 per cent ash, 1 to 10 per cent

TABLE 27  
*Influence of concentration of minerals in solution upon the nitrogen content of fungi*

MINERAL CONTENT OF MEDIUM	NITROGEN CONTENT OF DRY MYCELIUM
<i>per cent</i>	<i>per cent</i>
0.2	2.0
1.7	5.7
2.7	6.4

fat and 37 to 82 per cent carbohydrate. The nitrogen content of invertebrates has been reported<sup>34</sup> as 10.65 to 11.2 per cent for insects and insect larvae, 9.4 per cent for earthworms, and 4.9 per cent for myriapods.

The mineral composition of microorganisms depends also on the

<sup>31</sup> Peterson, W. H., Fred, E. B. and Schmidt, E. G. Jour. Biol. Chem., 54: 19-34. 1922.

<sup>32</sup> Raulin. J. Ann. Sci. Nat. Bot. (5), 11: 93. 1869.

<sup>33</sup> Zopf. Pilze. Breslau. 1890, p. 119-121. A detailed review of the chemical composition of fungi is given by Zellner, 1907 (p. 363).

<sup>34</sup> Morris, 1922 (p. 327).

<sup>35</sup> Cramer, E. Arch. Hyg., 28: 1-15. 1897.

mineral composition of the medium. It has been shown,<sup>35</sup> in the case of the cholera bacterium cultivated in nutrient bouillon, that, with an ash content of 2.1 per cent  $P_2O_5$  in the medium, the ash of the bacterial cells consisted of 10.9 per cent  $P_2O_5$ ; with 7.9 per cent  $P_2O_5$  in the medium, the ash of the bacteria contained 28.7 per cent  $P_2O_5$ ; with 39.8 per cent  $P_2O_5$  of the ash in the medium, the ash of the bacteria consisted of 38.4 per cent  $P_2O_5$ . The same is not true, however, of the other ash constituents. The latter include K, Na, Mg, Ca, Fe, Si, S and Cl, as shown in table 28. Some organisms (*Aspergillus*, *Penicillium*, *Mucor* and other fungi) can thrive without calcium, while others (*Azotobacter*) do not develop in the absence of this mineral element.<sup>36</sup>

TABLE 28  
*Mineral composition of microorganisms (de Rossi)*

	MINIMUM AND MAXIMUM FIGURES ON BASIS OF ASH CONTENT				
	Bacteria	Fungi	Spores of fungi	Yeasts	Higher fungi
	per cent	per cent	per cent	per cent	per cent
$P_2O_5$ .....	10.0-55.2	44.8-59.4	39.6	51.0-59.0	40.0
$SO_3$ .....	1.0- 8.0	Tr- 2.1	2.0	0.6- 6.0	8.0
$SiO_2$ .....	0.5- 7.8?	Tr- 2.0	....	0- 1.6	1.0
Cl.....	2.3-44.0	0.3	....	0.03- 1.0	....
$K_2O$ .....	4.0-25.6	8.7-39.5	46.0	28.0-40.0	45.0
$Na_2O$ .....	13.6-34.0	0.2-19.0	....	0.5- 1.9	1.4
MgO.....	0.1-11.5	3.8- 8.1	4.3	4.0- 8.1	2.0
CaO.....	0.3-14.0	1.0-13.8	....	1.0- 4.5	1.5
$Fe_2O_3$ .....	8.1	0.1- 0.7	5.0	0.1- 7.3	1.0

In the case of *Azotobacter*, there is a definite ratio between the nitrogen and phosphorus content of the cell. This relationship has been utilized in the development of a method for determining the amount of available phosphorus in the soil, as shown later (p. 707). A definite P:N relationship has also been found in the mycelium of fungi.<sup>37</sup>

The non-nitrogenous portion of the microbial cell consists chiefly of fats, gums and various hemicelluloses, chitin and frequently glycogen. Winterstein and Reuter<sup>38</sup> found that the ether extract of higher fungi amounted to 4.0 per cent of the total material (3.3 per cent fat and 0.5

<sup>35</sup> Loew, O. *Biol. Centrbl.*, **45**: 122-125. 1925.

<sup>37</sup> Schnücke, R. *Biochem. Ztschr.* **153**: 378. 1924; *Centrbl. Bakt.* II, **75**: 237. 1928.

<sup>38</sup> Winterstein, E. and Reuter, C. *Centrbl. Bakt.* II, **34**: 566-572. 1912; N. N. Iwanoff. *Biochem. Ztschr.*, **137**: 331-340. 1923.

per cent cholesterol<sup>39</sup>); the alcohol extract to 12 per cent (sugars, lecithin, bases, amino acids, purine bodies); water soluble portion, 28 per cent (sugars, glycogen, amino acids, purine bases, etc.); the residue was made up of 30 per cent protein, 10 per cent amorphous carbohydrate (para-iso-dextrin) and 6 per cent chitin. Fungi are capable of synthesizing soluble phosphatides,<sup>40</sup> as well as resins, higher alcohols<sup>41</sup> and a great variety of other substances.

The lipoids in the cell wall are primarily responsible for the difficulty with which bacterial proteins are digested. When gram-negative bacteria are treated with flowing steam or with lipid soluble substances, the digestibility is greatly increased.<sup>42</sup> Gram-positive bacteria (*Bac. cereus*, *Bac. subtilis*) are more easily oxidizable (isoelectric point at pH 2.0 to 3.0) than gram-negative bacteria (*Bact. coli*, *Bact. aerogenes*, isoelectric point at pH 5.0).

The place of glycogen in the cell may be taken by other reserve carbohydrates, as mycodextran and mycogalactan found in young cultures of *Pen. expansum* and *Asp. niger*.<sup>43</sup> The starch content of fungi was found to be influenced by the nitrogen-carbohydrate ratio of the medium: the lower the ratio, the greater is the starch formation in the cells of the fungi. Cramer<sup>44</sup> previously obtained from the spores of *Pen. glaucum* 17 per cent of "spore starch." This was, probably, an impure preparation of mycodextran contaminated by some other carbohydrate, giving the iodine reaction. Galactans have been reported in bacterial slime.<sup>45</sup> A number of hexosans, including cellulans related to cellulose and produced by *Bac. asteroides* and *Bact. prodigiosum*, levulans formed by bacteria belonging to the *Bac. subtilis* group, and dextrans produced by *Bact. radicola*, have been reported in bacterial slime.

<sup>39</sup> Various fungi and yeasts are known to produce a number of sterols (Hartmann, E. and Zellner, J. Monatsch. 50: 193-200. 1928). They serve, therefore, as excellent sources of ergosterol.

<sup>40</sup> Grafe, V. and Magistris, H. Biochem. Ztschr., 162: 366-398. 1925; Vorbrodt, W. Acta. biol. exper. Warsaw, 1: No. 5, 1928.

<sup>41</sup> Pontillon, C. Compt. Rend. Acad. Sci., 188: 413-415. 1929; Obaton, F. Ibid. 188: 77-79. 1929.

<sup>42</sup> Dukes, C. E. Brit. Med. Jour. I, 430-432. 1922.

<sup>43</sup> Dox, A. W. and Neidig, R. E. Jour. Biol. Chem., 18: 167-175. 1914; 19: 235-237. 1914; 20: 83-85. 1915; Schmidt, D. Biochem. Ztschr., 158: 223-252. 1925.

<sup>44</sup> Cramer, E. Arch. Hyg., 20: 197-210. 1894.

<sup>45</sup> Schardinger, F. Centrbl. Bakt. II, 8: 144-147, 175-180. 1902; Beijerinck, M. W. Folia Microb., 1: 377-408. 1912; Kramer, E. Centrbl. Bakt. I, 87: 401-406. 1921.

According to Lemoigne,<sup>46</sup> certain spore-forming soil bacteria (*Bac. megatherium*) contain a polymer of  $\beta$ -oxybutyric acid ( $\text{CH}_2\text{-CHOH}\cdot\text{CH}_2\text{-COOH}$ ), which may make up 18 to 20 per cent of the bacterial cell, this substance being in the nature of a poly-lactide. The presence in bacterial cells of polysaccharides made up of sugar acids (glucuronic, aldobionic, galacturonic) and of sugars has also been demonstrated.<sup>47</sup> These are found largely in the capsular material of the cell. While the uronic acids in plants consist largely of galacturonic acid, the uronic acids (in the bacterial slimes and gums) of bacteria are rich in glucuronic acid.

The chemical composition of the spores of a fungus is given in table 29.<sup>48</sup>

TABLE 29  
*Composition of Asp. oryzae spores*

	PER CENT		PER CENT
Water.....	17.43	Protein nitrogen.....	3.64
Ether extract.....	0.88	Water-soluble nitrogen.....	6.93
Alcohol extract.....	23.25	Amine-N.....	0.94
Carbohydrate (as glucose)...	9.00	Phosphotungstic acid precipitated N.....	5.25
Water-soluble carbohydrate...	0.60	Ammonia-N.....	0.31
Pentose and methyl pentose.	0.66	Total phosphorus in ash	
Glycogen.....	6.33	( $\text{P}_2\text{O}_5$ ).....	66.05
Ash.....	5.33	Lecithin.....	1.32
Crude fiber.....	11.21	Ergosterol.....	Present
Total nitrogen.....	8.69		

*Antagonism and symbiosis among microorganisms.* The metabolic products of one microorganism may prove either beneficial and stimulating or injurious and destructive to other organisms. Some of the products of metabolism of one organism may actually be used as nutrients by another, as in the case of nitrites produced in the oxidation of ammonium salt by one bacterium and used as a source of energy by another, or the organic acids produced by certain fungi and bacteria from carbohydrates and used as sources of energy by other organisms. Some microorganisms may produce a change in reaction of the medium, in the

<sup>46</sup> Lemoigne, M. Ann. Inst. Past., 39: 144. 1925; 41: 148. 1927.

<sup>47</sup> Goebel, W. F. Jour. Biol. Chem., 74: 619. 1927; Heidelberger, M. and Goebel, W. F. Ibid., 70: 613. 1926.

<sup>48</sup> Sumi, M. Biochem. Ztschr., 195: 161-174. 1928; 204: 412-413. 1929.



oxygen tension, concentration of nutrients, etc., which may be favorable to some organisms and unfavorable to others. Lactic and butyric acid bacteria, nitrifying and sulfur-oxidizing bacteria may produce enough acid so as to injure the development of proteolytic bacteria, of *Azotobacter* and of nitrifying bacteria; aerobic microorganisms may consume enough oxygen so as to make conditions favorable for anaerobic bacteria. Some investigators explain the nature of antagonism that exists between certain organisms on the basis of physico-chemical changes produced in the medium.<sup>49</sup>

The formation by one organism of toxic substances which are injurious to others has been frequently observed in the case of bacteria (*Bact. fluorescens* against spore-forming bacteria and micrococci),<sup>50</sup> actinomycetes and fungi grown on artificial culture media. It still remains to be established to what extent this is of importance in soil processes. The same is true of the production of vitamins (so-called auximones) by bacteria<sup>51</sup> and their influence upon the growth of other microorganisms and higher plants.

The existence of numerous associations between microorganisms and higher plants, as in the case of leguminous plants and mycorrhiza forming plants, between bacteria and animals, as in the digestion of cellulose by herbivorous animals, has been definitely established. The associations between microorganisms are still little understood. It is sufficient to point to the following illustrations: the associative growth of the nitrogen-fixing *Azotobacter* and the chlorophyll-bearing algae, in which the former fixes the nitrogen and the latter supplies the necessary energy; the mutualistic growth of aerobic and anaerobic bacteria in which the first use up the oxygen making conditions favorable for the activities of the second; in many cases the anaerobic bacteria can decompose cellulose producing organic acids which can be used as sources of energy by the aerobic organisms; decomposition of cellulose by aerobic bacteria in the presence of nitrate and bacteria capable of reducing the nitrate, the former supplying an energy source and the latter a source of oxygen. The associative growth of protozoa and algae is well illustrated in *Paramoecium bursarii*,<sup>52</sup> in the light and in the presence

<sup>49</sup> Arnaud, C., Kopaczewski, W. and Rosnowski, M. Compt. Rend. Acad. Sci., 185: 153-156. 1927.

<sup>50</sup> Lewis, I. M. Jour. Bact., 17: 89-103. 1929.

<sup>51</sup> Scheunert, A. and Schierblich, M. Liebig's Ann., 453: 249-258. 1927; Biochem. Ztschr., 184: 58-66. 1927.

<sup>52</sup> Pringsheim, E. G. Arch. Protistenk., 64: 289-418. 1928.

of available nitrogen, the algae, living within the cells of the protozoan, use the photosynthetic energy of the sun for the building up of organic complexes; in the digestion of the algae by the protozoan, the cell contents of the algae are brought into solution and the empty cell walls are excreted. Numerous other instances are known of the living together of two different organisms, which results in benefit to both or chiefly to one of the two.

Among the antagonistic effects of one organism upon another, we may include, in addition to the production of unfavorable conditions for growth (change in reaction, oxygen tension, consumption of nutrients) and production of actual toxic-like substances, the actual digestion of one organism by another. It is sufficient to cite the feeding of protozoa upon various groups of bacteria, of predacious nematodes upon parasitic forms, etc. Here must be included also the disease producing organisms, ranging from the invisible bacteriophages and ultra-microscopic viruses to the large wood-destroying mushroom fungi and insects.

*Summary.* A knowledge of the principles of nutrition of microorganisms, formation of waste products and synthesis of microbial cell substance is essential to an understanding of the numerous biochemical processes carried out in the soil. A knowledge of the conditions influencing the growth of microorganisms, the rate of growth and changes produced in the substrate, and the mutual interrelations between different organisms will greatly assist in interpreting the rôle of the numerous organisms in soil processes.

## CHAPTER XVI

### DECOMPOSITION OF CARBOHYDRATES, FATS AND HYDROCARBONS BY MICROORGANISMS

*Composition of vegetable organic matter.* Vegetable organic matter is added to the soil in the form of green manure, stable manure, plant residues and roots of cultivated and wild plants. This organic matter consists largely of cellulose, hemicelluloses, including pentosans and pectins; of starch, inulin, glucosides and sugars; of lignins and tannins; of proteins, protein degradation products and purine bases; of fats, oils, waxes, resins, pigments and mineral matter. A typical analysis of a series of native vegetable substances is given in table 30, in per cent of water-free substance.<sup>1</sup> The difference in the analyses reported by different investigators for the same type of plant material is due to differences in the nature of the plant varieties, age of plant, methods of determination, errors in manipulation, etc.

The constituents of cell membranes can be generally classified into several groups.<sup>2</sup>

1. True cellulose, or the condensation products of glucose, not acted upon by dilute acids.

2. Hemicelluloses, or the condensation products of hexoses (galactose, mannose), of pentoses (arabinose, xylose), of sugar acids or uronic acids (glucuronic, galacturonic) or mixtures of these (pectins, gums), all of which are readily hydrolyzed by dilute mineral acids.

3. Lignins, or complex, benzol-ring containing substances, forming compounds with cellulose known as ligno-celluloses, which constitute the woody tissues of plants; these ligno-celluloses are either chemical compounds, or physical, adsorption complexes, or merely mechanical incrustations of lignin on the cellulose.

4. Corky or cutinized lamellae, identified microscopically by the Sudan-glycerol reaction and not well defined chemically.

<sup>1</sup> Pringsheim, H. *Die Polysaccharide*. 3d Ed. 1931, Berlin.

<sup>2</sup> Tollens, B. *Ber. deut. chem. Gesell.*, **34**: 1434-1441. 1901; Heuser, E. *Lehrbuch der Cellulosechemie*. 3 Aufl. Berlin, Borntraeger. 1928.

The woody parts of plants or the plant cell membranes are usually referred to as "crude fiber." This is made up of cell walls composed principally of cellulose, which is accompanied by various other substances, depending upon the nature of the plant. It was believed that cellulose of different origin varies in chemical composition;<sup>3</sup> this is probably due not to differences in the constitution of the cellulose, but to differences in the amount and kind of impurities present. In the case of cotton, flax and hemp, pectin forms the chief impurity; in the case of straw, wood and jute, lignin is the impurity. Cellulose of different origin is decomposed with different rapidity,<sup>4</sup> presumably as a result of differences in the physical condition as well as the impurities present. The structure of the carbohydrate influences profoundly the way in which a micro-organism breaks up a molecule and the end products of the reaction.<sup>5</sup>

TABLE 30  
*The percentage composition of a few plant materials*

	CELLULOSE	PENTOSANS	LIGNIN	CRUDE PROTEIN	GUMS AND WAXES	ASH
Hay.....	28.50	13.52	28.25	9.31	2.00	6.05
Oat straw.....	35.43	21.33	20.40	4.70	2.02	4.81
Barley straw.....	32.92	21.45	18.66	3.20	1.40	5.56
Corn cobs.....	37.66	31.50	14.70	2.11	1.37	1.80
Corn fodder.....	30.56	23.54	15.13	3.50	0.77	6.15
Rice straw.....	31.99	27.67	18.48	5.33	0.51	5.43
Straw of winter cereals.....	34.27	21.67	21.21	3.00	0.67	4.33

In the study of decomposition of the various plant constituents, none received greater consideration than cellulose.

*Chemistry of cellulose.* Chemically, cellulose is a non-nitrogenous, amorphous polysaccharide, exhibiting a characteristic fibrous structure. It is insoluble in simple solvents and soluble in ammoniacal copper solution, in  $\text{ZnCl}_2$  and in strong acids ( $\text{H}_2\text{SO}_4$ ), giving a dark brown to violet color with chlor-zinc iodide. It is very resistant to the action of plants, animals and majority of microorganisms, but can be hydrolyzed by strong acids and by certain specific microorganisms. Cellulose

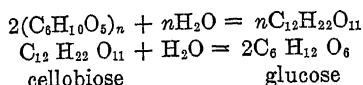
<sup>3</sup> König, J. Z. Unters. Nahrungs. u. Genussm., **12**: 388. 1906; Ber. deut. chem. Gesell., **39**: 3564-3570. 1906.

<sup>4</sup> Ernest, A. Ber. deut. chem. Gesell., **39**: 1947-1951. 1906.

<sup>5</sup> Peterson, W. H., Fred, E. B. and Marten, E. A. Jour. Biol. Chem., **70**: 309-317. 1926.

occurs only in a natural state (plant tissues) and has not as yet been synthesized in the laboratory. Cotton contains 87 to 91 per cent cellulose; wood of evergreens, 45–50 per cent; and cereal straw, 35 per cent. The empirical formula for cellulose is that of polysaccharides, namely  $(C_6H_{10}O_5)_n$ , the same as that of starch. It contains 44.42 per cent carbon and 6.22 per cent hydrogen. The ratio of oxygen to hydrogen is 8:1. On hydrolysis of cellulose, simple carbohydrates with an aldehyde (d-glucose united by cellobiose linkages) grouping are obtained.

Various formulae have been suggested to account for the chemical structure of the cellulose molecule. This is looked upon either as a polymerized molecule of cellobiose held together by strictly primary valences or as a colloid molecule held together by secondary or residual valences. When hydrolyzed by means of acetic acid anhydride and sulfuric acid, the cellulose swells and goes into solution in the form of a colloidal cellulose slime. On further hydrolysis, the disaccharide cellobiose is obtained (only about 36 per cent).

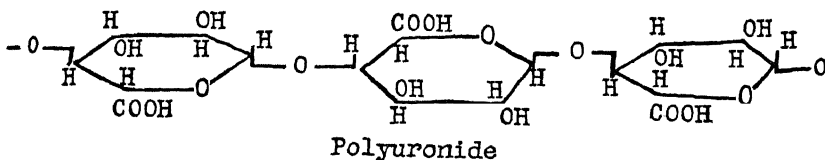
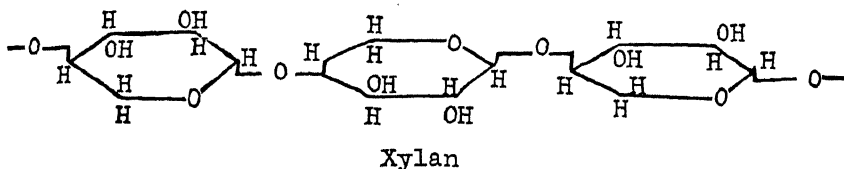
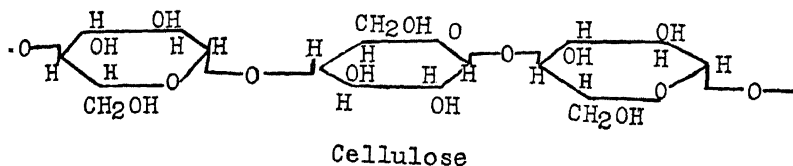


The existence of a reducing trisaccharide (procellose) as a possible intermediary product of hydrolysis between cellulose and cellobiose has been suggested.<sup>6</sup> On hydrolysis, it gives a molecule of glucose and one of cellobiose, the latter breaking down to two molecules of glucose. On the basis of X-ray investigations, the cellulose complex is considered to consist of four anhydro-glucose units. Sponsler and Dore,<sup>7</sup> as a result of a study of the Röntgen diagram of Ramie fibers, found cellulose to consist of glucose units in the form of amylene oxide rings, apparently united by primary valences in chains of indefinite length; they considered a group of eight glucose molecules as the simplest unit to represent the chemical structure of cellulose.

<sup>6</sup> Bertrand, G. and Benoist, S. *Compt. Rend. Acad. Sci.*, **176**: 1583–1587. 1923. Further information on the chemistry of cellulose is given by Schwalbe, C. G. *Die Chemie der Cellulose*. Berlin, Borntraeger. 1911; Czapek, F. *Biochemie der Pflanzen*. Bd. 1, p. 645; Heuser, 1928 (p. 372); Pringsheim, 1931 (p. 372); Euler, A. C. *Chem. Ztg.*, **45**: 977–998. 1921; Hibbert, H. *Jour. Ind. Engin. Chem.*, **13**: 256–260; 334–342. 1921; Cross, C. F. and Bevan, E. J. *Cellulose*. Longmans, Green & Co., London. 1916; Karrer, P. *Einführung in die Chemie der polymeren Kohlenhydrate*. Akad. Verlag. Leipzig. 1925; Hess, K. *Die Chemie der Cellulose und ihrer Begleiter*. Akad. Verlag., Leipzig. 1928.

<sup>7</sup> Sponsler, O. L. and Dore, W. H. *Colloid Symp. Monogr.* 1926, 174–202.

Structurally, cellulose has been presented as follows, whereby its chemical relationship to xylan and polyuronides is clearly indicated:<sup>7a</sup>



For a quantitative determination of cellulose in soil and in plant materials, several methods are available. The chlorination method used by the wood chemist does not give pure cellulose and the results cannot be, therefore, well interpreted. A more reliable method is that of Charpentier.<sup>8</sup>

Schweitzer's reagent is prepared by dissolving 200 grams  $\text{CuSO}_4$  in hot water, then precipitating with a calculated amount of ammonia (23 grams  $\text{NH}_3$  or 99 cc. of ammonia water, specific gravity 0.90). The excess of ammonia is neutralized with sulfuric acid. The precipitate is washed 3-4 times by decantation, then filtered on a Buchner funnel through a hardened filter paper. The hardened precipitate is dissolved in sufficient ammonia water (specific gravity 0.91), by shaking for 4 to 5 hours, so that 100 cc. of reagent contains 1.5 grams Cu (5 cc. of reagent is evaporated over sulfuric acid under a bell-jar, dried and heated to constant weight and weighed as  $\text{CuO}$ ). Twenty grams of soil previously treated with finely divided cellulose are placed in a cylinder of 150 cc. capacity with 100

<sup>7a</sup> Meyer, K. H. *New Phytologist*, **30**: 1-10. 1931.

<sup>8</sup> Charpentier, C. A. G. Thesis. Univ. Helsingfors. 1921; Meddel. No. 205 Centralanst. forsoksv. jordbruck. Bakteriell. Avdel. No. 22, Stockholm. 1920.

cc. of the Schweitzer reagent. The cylinder is closed and shaken, in a special shaking machine, for 30 minutes; the suspension is then allowed to settle. The solution is filtered through asbestos in a Gooch crucible and 50 cc. of the filtrate are precipitated with 200 cc. of 80 per cent alcohol. The precipitate is filtered through a Gooch crucible and is then washed with (1) dilute (1 per cent) hydrochloric acid, (2) distilled water, (3) dilute (2 per cent) KOH solution, (4) distilled water, (5) dilute HCl solution, (6) distilled water, (7) alcohol, and finally (8) ether. The cellulose is then dried at 100° to 110° to constant weight, burnt off and the crucible is reweighed. The difference obtained gives the amount of cellulose in 10 grams of soil.

Soils rich in organic matter (so-called "humus soils") have to be treated first with 10 per cent CaO, so as to neutralize the humus substances, which would prevent the solution of the cellulose,<sup>9</sup> or have to be extracted with a 2 per cent solution of sodium hydroxide, then washed with water, dilute acetic acid and again with water. To determine cellulose in straw, wood or other plant materials added to the soil, the latter is first treated with sodium acid sulfite, and then extracted with Schweitzer's reagent;<sup>10</sup> the plant material may also be extracted with 2 per cent sodium hydroxide solution, at 15 pounds pressure, for 30 to 60 minutes, then washed and boiled with 2 per cent solution of sulfuric acid and washed, and only then extracted with Schweitzer's reagent; the precipitation of the cellulose, filtering, washing and igniting is carried out as in the case of pure cellulose.

This method as well involves considerable difficulty and errors in manipulation. A much simpler method of determination of cellulose in plant materials consists in treating the material with ether and hot alcohol, then with a 2 per cent solution of hydrochloric acid, at 100°C. for 3-5 hours. The residue is filtered, washed and dried. The dried and weighed residue is treated with 10 volumes of 80 per cent sulfuric acid for 2 to 2½ hours in the cold. The material is then treated with 15 volumes of water and boiled for 5 hours. The digest is filtered and an aliquot portion of the filtrate is used for determination of reducing sugar. Theoretically 100 units of cellulose should give 110 parts of sugar. The amount of sugar found is then multiplied by 0.9 to give the cellulose content. Actually only about 95 per cent of the theoretical amount of cellulose is obtained by this method.<sup>11</sup>

*Mechanism of decomposition of cellulose by microorganisms.* Mitscherlich<sup>12</sup> was the first (1850) to attribute the fermentation of cellulose

<sup>9</sup> Dmochowski, R. and Tollens, B. Jour. Landw., 58: 1-20. 1910.

<sup>10</sup> Bengtsson, N. Meddel. Centralanst. Bakt. Avd. 37. 1925.

<sup>11</sup> Ost and Wilkening. Ber. deut. Chem. Gesell., 46: 2995. 1913; Kiesel, A. and Semiganowski, N. Sci. Chem. Pharm. Inst. Moskau, 18: 83-94. 1927; Waksman, S. A. and Stevens, K. R. Soil Sci., 26: 113-137. 1928; Jour. Ind. Engin. Chem. Anal. Ed., 2: 167. 1930.

<sup>12</sup> Mitscherlich, E. Ber. Bekanntmach. Verhandl. Königl. Preuss. Akad. Wissensch. Berlin, 1850, 102-110.

to the activities of microorganisms. Popoff<sup>13</sup> demonstrated in 1875 the connection between cellulose decomposition and methane formation; in the anaerobic decomposition of organic matter, the  $\text{CO}_2:\text{CH}_4$  ratio was found to be 1:1. Tappeiner<sup>14</sup> established conclusively that microorganisms are concerned in the decomposition of cellulose.

In studying the bacterial changes which take place normally in the intestinal canal, Tappeiner introduced finely divided cotton or paper into flasks containing a 1 per cent neutral solution of beef extract. The flasks and contents were sterilized and then inoculated with small quantities of pancreatic juice and incubated at 35°C. They were so arranged that the gases could be collected and analyzed. The decomposition products consisted of acetic acid, isobutyric acid, acetaldehyde, methane and carbon dioxide. The last two were in the ratio of 1:7.2 at the beginning of the experiment and 1:3.4 at the close. In another set of experiments an alkaline medium was used; the same qualitative but different quantitative results were obtained, there being a large amount of hydrogen evolved in the alkaline medium.

Hoppe-Seyler<sup>15</sup> placed 25.773 grams of filter paper into 1000 cc. flasks containing 700 cc. of water; this was inoculated with sewage and the gaseous products collected over mercury. The cultures were incubated at room temperature for four years. During the first year there was considerable gas evolved, but the evolution gradually became slower until, at the end of four years, it had practically ceased. The analysis showed that 15 grams of the cellulose had been decomposed with the formation of carbon dioxide and methane. Hoppe-Seyler was unable to find among the products any true sugars, although he thought it possible that there were some dextrin compounds in solution. When air was excluded, there was a greater production of methane and less carbon dioxide. Hoppe-Seyler suggested that the process of cellulose decomposition proceeds in two stages: the cellulose is first hydrated with the formation of glucose, according to the equation:



The glucose was believed to be broken down to carbon dioxide and methane:



<sup>13</sup> Popoff, L. Arch. Ges. Physiol., 10: 113-146. 1875.

<sup>14</sup> Tappeiner, H. Ber. deut. chem. Gesell., 15: 999-1002. 1882; Ztschr. Biol., 20: 52-134. 1884.

<sup>15</sup> Hoppe-Seyler, F. Ztschr. physiol. Chem., 10: 201-217; 401-440. 1886.



Omeliansky<sup>16</sup> distinguished between methane and hydrogen fermentations of cellulose, both processes being anaerobic in nature; organic acids, alcohols and gases were also formed. Van Iterson<sup>17</sup> was the first to demonstrate that cellulose decomposition may also take place under aerobic conditions. Aerobic and anaerobic decomposition of cellulose were considered to be two distinct processes, carried out by different organisms and under distinctly different conditions.

The microorganisms capable of decomposing cellulose can be divided into six general groups: (1) anaerobic bacteria, (2) aerobic bacteria, (3) filamentous fungi, (4) higher or mushroom fungi (Basidiomycetes), (5) actinomyces, and (6) possibly also protozoa and other invertebrate animals.<sup>18</sup> In normal cultivated soils, the aerobic bacteria, fungi, and actinomyces are largely concerned with the decomposition of cellulose.<sup>19</sup> Under anaerobic conditions, as in peat bogs, anaerobic bacteria are responsible for the decomposition of cellulose; while, in the manure heap, thermophilic bacteria may be largely active. Cellulose is digested by the herbivorous animals by means of the bacteria present in their intestinal tract.<sup>20</sup> The same is probably true of the digestion of cellulose by insects.<sup>21</sup>

Some bacteria decompose cellulose with the formation of a clear zone around the colony, which is believed to indicate the formation of an exocellular enzyme.<sup>22</sup> Pringsheim<sup>23</sup> allowed the decomposition of cellulose to proceed till a maximum has been reached, as indicated by gas formation. Bacterial action was then quickly brought to a standstill by the introduction of a proper antiseptic or a change in temperature, which prevented the further development of the bacteria without injuring the enzyme. Sugars, as the products of hydrolysis, accumulated, due to the fact that the sudden stop of bacterial action did not prevent the hydrolytic enzyme (*cellulase*) from breaking down the cellulose. The hydrolytic products, cellobiose and glucose, were demonstrated

<sup>16</sup> Omeliansky, 1902 (p. 186).

<sup>17</sup> Iterson, G. van. Centrbl. Bakt. II, 11: 689-698. 1904.

<sup>18</sup> Younge, C. M. Sci. Progr. No. 78: 242-248. 1925.

<sup>19</sup> Mütterlein, C. Inaug. Diss. Leipzig. 1913; Waksman and Skinner, 1926 (p. 185); Pringsheim, H. Centrbl. Bakt. II, 37: 111-112. 1913.

<sup>20</sup> Scheunert, A. Ztschr. physiol. Chem., 48: 9-26. 1908; Hösslin, A. and Lesser. Ztschr. Biol., 54: 47. 1910.

<sup>21</sup> Werner, E. Centrbl. Bakt. II, 67: 297-330. 1926.

<sup>22</sup> Kellerman and McBeth, 1912 (p. 192). Löhnis, F. and Lochhead, A. G. Centrbl. Bakt. II, 58: 430-434. 1923.

<sup>23</sup> Pringsheim, H. Ztschr. physiol. Chem., 78: 266-291. 1912.

by the reduction of Fehling's solution, and formation of corresponding osazones. Since the decomposition of cellulose is a comparatively slow process, especially at normal temperatures, the hydrolytic action of the enzyme is also very slow and does not lead to any abundant accumulation of products of hydrolysis. This, as well as the destruction of the hydrolytic enzymes by proteolysis, suggested the use of large quantities of media which are concentrated in vacuo, at a low temperature, so as to obtain a solution with a sugar concentration sufficient for identification. In the anaerobic and denitrifying processes, it takes two to seven days before reducing sugar can be demonstrated. The thermophilic bacteria, which are much more active, give a strong reduction of Fehling's solution in 24 hours.<sup>24</sup>

Just as starch is hydrolyzed by amylase at first to the disaccharide maltose and then by a separate enzyme (maltase) to glucose, so is cellulose first hydrolyzed by the cellulase of bacteria to the disaccharide cellobiose, then by the enzyme cellobiase to glucose. The latter enzyme has also been demonstrated in cellulose decomposing bacteria and fungi.<sup>25</sup> In an alkaline medium, cellobiose is formed faster than it is hydrolyzed.<sup>26</sup>

The glucose produced from cellulose by the action of the bacterial enzymes is rapidly broken down by the same organisms, especially under anaerobic conditions or by accompanying bacteria, to various organic acids, such as acetic, butyric and lactic or formic, acetic and valeric. These acids are decomposed, either by the same bacteria or by a secondary flora, to carbon dioxide and water. In some cases, the transformation of the cellulose leads to the formation of mucilages consisting of hemicelluloses. According to Neuberg and Cohn,<sup>27</sup> acetaldehyde is formed as an intermediary product in the decomposition of cellulose by thermophilic bacteria. It is this acetaldehyde which may serve as

<sup>24</sup> See also Peterson, W. H., Scott, S. W. and Thompson, W. S. *Biochem. Ztschr.*, **219**: 1-6. 1930.

<sup>25</sup> Fischer, E. and Zemplén, G. *Liebig's Ann. Chem.*, **365**: 1-6. 1909; **372**: 254-256. 1910; Bertrand, G. and Holderer, M. *Compt. Rend. Acad. Sci.*, **149**: 1385. 1909; **150**: 230. 1910.

<sup>26</sup> Groenewege, J. *Bull. Jard. Bot. Buitenzorg* (3), **2**: 287. 1920; Meddel. Alg. Proefsta. Landbr. Dept. Nijv. Handel., **13**: 1-23. 1923; For the formation of cellulose-decomposing enzymes by fungi, see Kellerman, K. F. *U. S. Dept. Agr. Bur. Pl. Ind. Circ.* 113, 1912; Ellenberger, W. *Ztschr. physiol. Chem.*, **96**: 236-254. 1915; Kosin, N. I. *Rpt. of the Physico-Chem. Lomonossov Soc.*, Moskau, **2**: 57-98. 1921.

<sup>27</sup> Neuberg, C. and Cohn, R. *Biochem. Ztschr.* **139**: 527-544. 1923.

the building stone for the synthesis of the microbial protoplasm. The mechanism of decomposition of cellulose by microorganisms still represents a number of unsolved complicated problems.

*Decomposition of cellulose by anaerobic bacteria.* The anaerobic decomposition of cellulose results in the formation of organic acids and gases consisting of carbon dioxide and methane or hydrogen. When cellulose was decomposed, in Omeliansky's experiments<sup>28</sup> by the methane organism, 50 per cent of the substrate was changed to gas (6.5 per cent methane and 43.5 per cent CO<sub>2</sub>) and 50 per cent into fatty acids (acetic and butyric); the hydrogen organism changed about 33 per cent of the substrate into gases (4 per cent hydrogen and 29 per cent CO<sub>2</sub>) and 67 per cent into fatty acids (acetic, butyric, and small quantities of valerianic acid). The decomposition of cellulose is soon stopped by the rapid accumulation of the acids, unless CaCO<sub>3</sub> is added to keep the medium neutral. Even then the process of cellulose decomposition is very slow and it may take months before 5 to 10 grams of filter paper suspended in a liter of medium are fully dissolved. The hydrogen organism decomposed in thirteen months 3.347 grams of cellulose, with the formation of,

	<i>grams</i>
Fatty acids.....	2.240
CO <sub>2</sub> .....	0.972
H <sub>2</sub> .....	0.014

The loss of 0.121 gram was believed to be due to substances not determined, including valerianic acid, higher alcohols, aromatic substances and dissolved hydrogen.

The methane organism formed, out of 2 grams of cellulose decomposed:

	<i>grams</i>
Fatty acids.....	1.022
CO <sub>2</sub> .....	0.868
CH <sub>4</sub> .....	0.137

A convenient apparatus for the study of cellulose decomposition under anaerobic conditions is shown in fig. 16.

The anaerobic bacillus isolated by Khouvine<sup>29</sup> from the human intestinal tract was capable of using cellulose as the only source of energy;

<sup>28</sup> Omeliansky, 1904 (p. 186).

<sup>29</sup> Khouvine, 1923 (p. 188).

sugars could not be utilized. One gram of cellulose disappeared in 16 days; but, in the presence of other bacteria, five times as much cellulose was decomposed. Among the decomposition products,  $\text{CO}_2$ ,  $\text{H}_2$ , ethyl alcohol, acetic and butyric acids, traces of lactic acid, products precipitated by alcohol and a yellow pigment could be demonstrated.

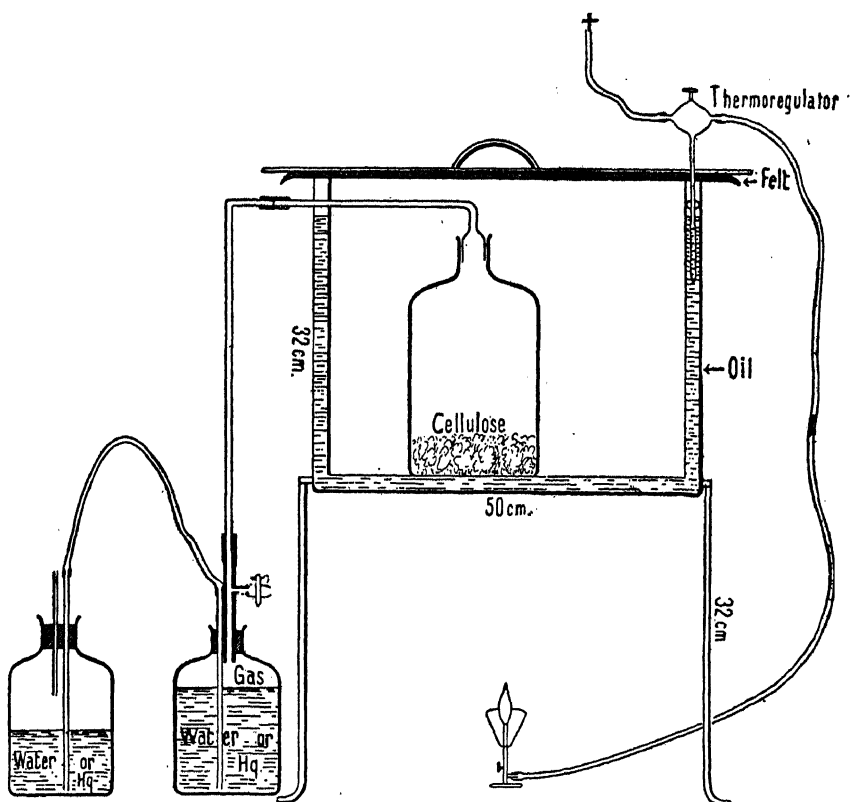


FIG. 16. Apparatus for study of cellulose decomposition, under anaerobic conditions (after Pringsheim).

In the case of the thermophilic cellulose-decomposing bacteria, a nutrient solution containing pure filter paper is inoculated with a small quantity of soil and incubated at  $60^\circ\text{C}$ . The filter paper is disintegrated in 10 to 14 days, with the formation of  $\text{CO}_2$  and  $\text{CH}_4$ ; formic and acetic acids were also demonstrated among the decomposition prod-

ucts.<sup>30</sup> Organic nitrogen was found to be the best source of nitrogen.<sup>31</sup> Pringsheim<sup>32</sup> obtained, out of 3 grams of cellulose decomposed, 0.2125 gram formic acid, 1.15 gram acetic acid and a small quantity of lactic acid; carbon dioxide made up 21.9 to 49.1 per cent of the gases, the rest was hydrogen. By changing the conditions of growth, the relative amounts of the products may be changed. Out of 60 grams of cellulose added to 4 liters of medium containing 20 grams of peptone, 42 grams were decomposed by thermophilic bacteria with the formation of the following products:<sup>33</sup>

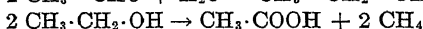
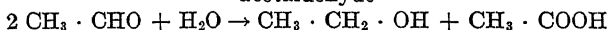
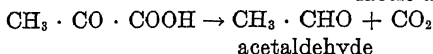
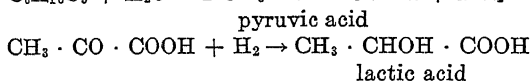
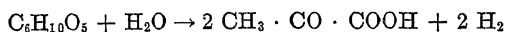
	YIELD	CARBON CONTENT
	grams	grams
Acetic acid.....	21.6	8.6
Alcohol.....	10.3	5.4
CO <sub>2</sub> .....	11.94	3.0

Carbon of cellulose decomposed..... 18.6 grams

Carbon of products accounted for..... 17.0 grams

Among the gases, hydrogen was formed in considerable quantities, but no methane.

The following equations were suggested<sup>34</sup> to explain the anaerobic decomposition of cellulose:



Lactic acid may also break down to give butyric acid



The rapid heating of hay results from transformation of cellulose and other carbohydrates in the hay by microorganisms.<sup>35</sup> *Bact. coli*,

<sup>30</sup> MacFayden and Blaxall, 1899 (p. 195).

<sup>31</sup> Kroulik, 1913 (p. 151).

<sup>32</sup> Pringsheim, 1913 (p. 195).

<sup>33</sup> Viljoen, Fred and Peterson, 1926 (p. 195).

<sup>34</sup> Lymn and Langwell. Jour. Soc. Chem. Ind., 42: 279-280. 1923.

<sup>35</sup> Mieke, 1907 (p. 287); Arb. deut. landw. Gesell., 196. 1911; (Centrbl. Bakt. II, 34: 281-282. 1912).

*Oidium lactis* and *Bac. calfactor* were found capable of heating up well packed hay and a mixture of two of these organisms could bring about a normal heating of moist hay. In addition to these, Miehe isolated an actinomyces and various fungi from the piles of heated hay. However, the actual rôle of these organisms in the heating of hay is still uncertain. It has been suggested that either inflammable products are formed or that the initial reaction of raising the temperature is biological, followed by chemical processes. Some investigators ascribe the process of heating of hay to the action of oxidative and reducing enzymes.<sup>36</sup>

*Decomposition of cellulose by aerobic bacteria.* Cellulose decomposition by aerobic bacteria can be studied by suspending filter paper in a shallow layer of a medium containing an appropriate nitrogen source and the necessary minerals and inoculating with a pure or crude culture of bacteria, with soil, or with manure. At room temperature, the cellulose will be found to be rapidly decomposed with the formation of a slime or mucilage which may be colored yellow or red. Carbon dioxide is formed abundantly but no other gas, so that no visible "fermentation" is found to take place; small quantities of acids are formed in the medium. The cellulose is largely macerated and the fibers separated from one another and gradually reduced to a pulp. Some organisms are very active and others are very slow. With some species isolated by Kellerman and associates,<sup>37</sup> the principal by-products were found to consist of formic and acetic acids, while others gave rise only to traces of fatty acids; no aldehydes, ketones or alcohols were formed.

The aerobic bacterium *Cytophaga Hutchinsoni* produces a pigment related to the carotin group, also some mucilage, which does not give rise to optically active compounds on hydrolysis, and small quantities of fatty acids, chiefly butyric. The mucilage is extracted in the crude "humus" fraction in soil analysis.<sup>38</sup> Winogradsky<sup>39</sup> considers this mucilage to be of the nature of oxycellulose produced as an oxidation product of cellulose.

In the decomposition of cellulose by denitrifying bacteria, the energy liberated is used for the reduction of nitrates to atmospheric nitrogen; the oxygen thus obtained is utilized for the oxidation of the cellulose.

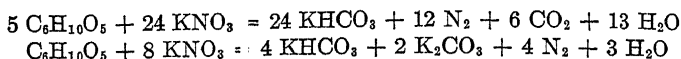
<sup>36</sup> Tschirch, A. Mitt. Naturf. Gesell. Bern. 138-152. 1917; Laupper, G. Landw. Jahrb. Schweiz, 34: 1-54. 1920; Matér. Étude Calam. 3(10): 112-140. 1927; Browne, C. A. Tech. Bul. 141, U. S. Dept. Agr. 1929; Curzi, M. Roy Staz. Pat. Veget. Rome, 10: 222-280. 1930.

<sup>37</sup> Kellerman et al., 1914-1916 (p. 192).

<sup>38</sup> Hutchinson and Clayton, 1918 (p. 190).

<sup>39</sup> Winogradsky, S. Compt. Rend. Acad. Sci., 187: 326-329. 1928.

Carbon dioxide and water are the chief products formed in the decomposition of cellulose, while nitrogen gas is a product of the reduction of nitrates. The formation of carbonates, due to the reduction of the nitrates, leads to the alkalization of the medium:



*Cellulose decomposition by actinomyces.* The capacity of decomposing cellulose and using it as a source of energy is well distributed among the actinomyces.<sup>40</sup> This can be readily demonstrated either by the cellulose-plate method or by adding cellulose, in the form of filter paper, to a medium containing the necessary inorganic salts and a source of nitrogen. Krainsky<sup>41</sup> found that certain pigment-producing species, forming small, spherical spores (*Act. melanocyclus* and *Act. melanosporus*) are particularly active in this connection, forming black or red rings on the paper and causing decomposition of the cellulose. On the agar plate, clear rings are formed around the colony, due to the decomposition of the cellulose by an exo-enzyme.

To study the process quantitatively, filter paper is placed in flasks containing a synthetic nutrient medium, with ammonium salt (plus calcium carbonate) or nitrate as a source of nitrogen. The paper is allowed to dip partly in the medium, since most of the actinomyces are aerobic organisms and do not develop readily below the surface of the medium. After incubation, the culture is filtered and residual paper washed and weighed. It is necessary to determine the nitrogen content of the washed paper, due to the presence of some of the mycelium of the organism. By allowing 10 per cent as the nitrogen content of the mycelium, the residual cellulose can be readily calculated. The liquid medium is analyzed for the residual inorganic nitrogen and various organic acids. A definite ratio exists between the amount of cellulose decomposed and nitrogen assimilated by the organism. Some of the actinomyces decompose the paper to such an extent as to leave transparent mucilaginous strands which fall to pieces when an attempt is made to lift them out of the medium. Red and black pigments may be produced through the paper. When compared with the bacteria and fungi capable of decomposing cellulose, the action of most of the actinomyces upon cellulose is only slow and limited.

*Cellulose decomposition by filamentous fungi.* Fungi act readily upon

<sup>40</sup> Fousek, 1913 (p. 289).

<sup>41</sup> Krainsky, 1913-1914 (p. 276).

cellulose and hemicelluloses of plant tissues.<sup>42</sup> Certain *Fusaria*, *Aspergilli*, *Penicillia*, *Trichodermae* and various *Dematiaceae* possess a strong cellulose-decomposing power. It has even been suggested that in normal cultivated soils, especially acid in nature, fungi play the most important rôle in the decomposition of cellulose in plant tissues. The following method can be used for comparing the cellulose decomposing power of different microorganisms:

Finely ground filter paper (1 gram) and 20 cc. of a nutrient solution (10 grams  $(\text{NH}_4)_2\text{SO}_4$ ,  $(\text{NH}_4)_2\text{HPO}_4$  or  $\text{NH}_4\text{NO}_3$ , 3 grams  $\text{K}_2\text{HPO}_4$ , 2 grams  $\text{MgSO}_4$ , 1 gram  $\text{NaCl}$ , 1000 cc. of tap water, with or without 5 grams  $\text{CaCO}_3$ ) are added to 100 gram portions of sand or soil, placed in flasks, sterilized (for 2 hours at 15 pounds pressure) and inoculated. If possible, it is advisable to connect the flasks with an aeration apparatus and determine the evolution of  $\text{CO}_2$ . When soil is used as a medium, a control without cellulose, sterilized and inoculated, should also be employed. When  $\text{CaCO}_3$  is added, allowance should be made for the  $\text{CO}_2$  given off by the interaction of organic acids with the carbonate. The cultures are incubated for 3 to 6 weeks at  $25^\circ$  to  $28^\circ\text{C}$ . At the end of the incubation period, the amount of cellulose left in an aliquot portion of the culture is determined. A series of results<sup>43</sup> obtained by this procedure, with sand as a medium, are presented in table 31.

There is good evidence that fungi are chiefly responsible for the decomposition of cellulose in acid, humid soils under aerobic conditions. When the soil is partially sterilized to eliminate the fungi, cellulose decomposition comes practically to a standstill. When cellulose is added to the soil, especially in the presence of an available source of nitrogen, the fungi develop much more abundantly than either the bacteria or actinomyces; this can be readily demonstrated both by the plate and direct microscopic methods.

*Cellulose decomposition by fleshy fungi or Basidiomycetes.* The ability of higher or fleshy fungi belonging to the Basidiomycetes to decompose cellulose readily is clearly illustrated in the phenomena of wood destruction. Most of the fungi attack the cellulose in wood and allow the lignin to accumulate, as in the processes known as "brown rot;" however, a few fungi, like *Polyporus annosus* and *Trametes pini*, are capable of attacking the lignin and cellulose as well and leave a residue consisting largely of cellulose, as in the so-called "white rots." The common cellulose decomposing fungi are found in the genera *Coniophora*,

<sup>42</sup> Schellenberg, 1908 (p. 247).

<sup>43</sup> Waksman, S. A. and Heukelekian, H. 4th Int. Conf. Pedology, Rome., 3: 216-228. 1924.



*Stereum*, *Formes*, *Lenzites*, *Merulius* (*M. lacrymans*), *Polyporus*, *Poly-stictus*, *Trametes*, *Poria* and *Armillaria* (*A. mellea*).<sup>44</sup> The common mushroom fungus (*Psalliota campestris*) is claimed<sup>45</sup> to be capable of decomposing cellulose, in the presence of inorganic nitrogen compounds and minerals. However, the chemistry of the processes involved is still little understood.

*Decomposition of cellulose by invertebrate animals.* The decomposition of cellulose in the digestive tract of worms, insects and other invertebrate animals is either carried out by the production of an

TABLE 81

*Decomposition of cellulose by pure cultures of microorganisms*

ORGANISM	INCUBATION	CELLULOSE DECOMPOSED
	days	per cent
<i>Trichoderma koningi</i> .....	21	46.8
<i>Trichoderma koningi</i> .....	42	95.0
<i>Fusarium</i> sp.....	21	36.8
<i>Aspergillus fumigatus</i> .....	21	93.0
<i>Aspergillus glaucus</i> .....	21	49.0
<i>Aspergillus wentii</i> .....	21	0
<i>Aspergillus fuscus</i> .....	21	0
<i>Penicillium</i> sp.....	21	8.5
<i>Mucor racemosus</i> .....	21	0
<i>Zygorhynchus mölleri</i> .....	21	0
<i>Cunninghamella elegans</i> .....	21	0
<i>Actinomyces violaceus-ruber</i> .....	21	6.8
<i>Actinomyces cellulosae</i> .....	42	12.8
<i>Actinomyces viridochromogenus</i> .....	42	0
<i>Bacterium fimi</i> <sup>46</sup> .....	42	29.0
<i>Bac. cereus</i> .....	42	0
<i>Bac. vulgatus</i> .....	42	0

enzyme cellulase<sup>47</sup> or by association with the bacteria inhabiting the digestive tract of the animals, as shown by Werner for the larvae of *Potosia cuprea* and by others.

<sup>44</sup> Thaysen and Bunker, 1927 (p. XIV).

<sup>45</sup> Styer, F. Amer. Jour. Bot. 15: 246-250. 1928.

<sup>46</sup> This organism of Kellerman et al., was from stock culture; *Cytophaga Hutchinsoni* was much more active and decomposed almost as much cellulose as the fungi.

<sup>47</sup> Boynton, L. C. and Miller, R. C. Jour. Biol. Chem., 75: 613-618. 1927.

It has been suggested<sup>48</sup> that certain protozoa are capable of decomposing cellulose and that the presence of protozoa in the intestinal tract of termites is responsible for the ability of these organisms to live on a cellulose diet; the relationship between the protozoa (*Trichonympha* and *Pyrosonympha*) and the host (*Reticulitermes flavipes*) is found to be one of symbiosis. It still remains to be definitely established, however, whether the protozoa are the actual cellulose-decomposing agents. The mechanism of cellulose decomposition by the shipworm, *Teredo navalis*, and other invertebrate animals also requires further elucidation.

*Cellulose decomposition and nitrogen-fixation.* Although cellulose cannot be used directly as a source of energy for nitrogen-fixing bacteria, it may form products which are available sources of energy for these organisms. Beijerinck<sup>49</sup> found that a medium consisting of two parts of paper, two parts of chalk and 0.05 per cent  $K_2HPO_4$  in 100 cc. of tap water was a favorable medium for nitrogen-fixation. At 25° to 30°, there developed chiefly the hydrogen-forming organism together with *Bact. radiobacter* and *Azotobacter*. For every gram of cellulose decomposed, 8 to 9 mgm. of nitrogen were fixed. *Cl. pastorianum* and *Azotobacter* are unable to attack cellulose in pure culture. Only in mixture with cellulose decomposing organisms, are they able to fix nitrogen, at the expense of the cellobiose, the glucose or the fatty acids formed from the decomposition of the cellulose. In a mixture of the methane-forming organism and *Clostridium americanum*, Pringsheim<sup>50</sup> obtained 12.1 mgm. nitrogen fixed for 1 gram of cellulose decomposed. The methane bacterium produced 10 grams fatty acid out of 20 grams cellulose; but in the presence of nitrogen-fixing organisms, only 0.064 gram fatty acid accumulated. *Azotobacter* fixed 4.5 mgm. of nitrogen out of 1 gram of cellulose, in symbiosis with the methane organism; this is probably due to the fact that *Azotobacter* does not thrive well under semi-anaerobic conditions. Under aerobic conditions, Koch<sup>51</sup> obtained a more favorable nitrogen fixation by *Azotobacter*.

Koch found stronger cellulose-decomposing bacteria in manure than in normal soil and, therefore, suggested that the beneficial effect of stable manure is due to the introduction of strong cellulose decomposing

<sup>48</sup> Cleveland, L. R. Biol. Bull., **46**: 177-225. 1924; **54**: 231-237. 1928.

<sup>49</sup> Beijerinck, M. W. Arch. Neerland. Sci. Ex. Nat. Ser. II, **9**: 8-36. 1904.

<sup>50</sup> Pringsheim, H. Centrbl. Bakt. II, **23**: 300-304. 1909; **26**: 222-227. 1910; Mitt. deut. landw. Gesell., 1912, 1913, p. 26, 43 (Centrbl. Bakt. II, **37**: 111. 1913).

<sup>51</sup> Koch, A. Centrbl. Bakt. II, **27**: 1-7. 1910; **31**: 567-577. 1911; Jour. Landw., **55**: 355-416. 1907.

bacteria, as shown by the increased action of green manure when it is inoculated with a small amount of stable manure. The more rapid decomposition of the green manure results in an increased nitrogen fixation. Similar ideas on the favorable influence of small amounts of stable manure have been expressed by Lipman and associates.<sup>52</sup> Their ideas were not confirmed by a careful analysis of the processes of cellulose decomposition. More recent information<sup>53</sup> tends to indicate that the favorable addition of small amounts of stable manure is due to the nutrients, especially the nitrogen that it contains.

Nitrogen-fixation resulting from the symbiotic action of nitrogen-fixing and cellulose-decomposing bacteria, with cellulose as the only source of energy, has been demonstrated by other investigators.<sup>54</sup> Peat is very resistant to the action of cellulose decomposing bacteria, but this resistance can be overcome by preliminary treatment of the peat, as boiling, steaming, or grinding. It is claimed that it may then become a source of energy for nitrogen fixing microorganisms.<sup>55</sup> The importance of this process in increasing the supply of soil nitrogen is, however, still questionable.

*Influence of soil conditions upon cellulose decomposition.* The existence of thermophilic bacteria in the soil indicates that a high temperature is not injurious to cellulose decomposition but may even be highly beneficial. According to Bertrand and Compton,<sup>56</sup> 46° is the optimum temperature for the action of cellobiase, while Pringsheim found that cellulose decomposition will take place at temperatures of 20° to 70°.

As to the influence of soil reaction, Hellström<sup>57</sup> demonstrated in 1899 that the action of lime on peat soils is not so much to serve as a nutrient for plants as to stimulate the decomposition of the organic substances in the peat and neutralize the acid products formed. Christensen<sup>58</sup> also found that a basic soil shows a much higher cellulose decomposition than a base-free soil. The results of Charpentier and Barthel seem to prove that liming of soil has no favorable action at all upon cellulose

<sup>52</sup> Lipman, J. G., Blair, A. W., Owen, I. L. and McLean, H. C. N. J. Agr. Exp. Sta. 25th. Ann. Rpt.: 248-260. 1912; 26th: 474-478. 1913; 27th: 223-226. 1914.

<sup>53</sup> Barthel, Chr. and Bengtsson, N. Soil Sci., 18: 185-200. 1924; Medd. No. 300, Centralanst. forsoks. jordbruks. Bakt. avd. 40. Stockholm. 1926.

<sup>54</sup> Hutchinson and Clayton, 1918 (p. 190); Groenewege, 1920 (p. 197).

<sup>55</sup> Schmidt, E. W. Centrbl. Bakt. II, 52: 281-289. 1920.

<sup>56</sup> Bertrand, G. and Compton, A. Bull. Soc. chim. France, (4), 9: 100. 1911.

<sup>57</sup> Hellström, P. 1909 (Ref. Charpentier, 1921, p. 375).

<sup>58</sup> Christensen, 1915 (p. 507).

decomposition. This is due largely to the fact that the process is carried out both by fungi and bacteria. The reaction of the soil influences the type of organisms taking an active part in cellulose decomposition, but not the process itself. Since various fungi and various bacteria decompose cellulose actively, a change in reaction will favor the development of either one group or another, while the actual amount of cellulose decomposed may be influenced only inappreciably. Jensen<sup>59</sup> demonstrated that at pH 6.5–7.0, the cellulose decomposing vibrios are active; at pH 5.7–6.3 the members of the *Cytophaga* group are active; at a greater acidity, the cellulose-decomposing fungi are most abundant.

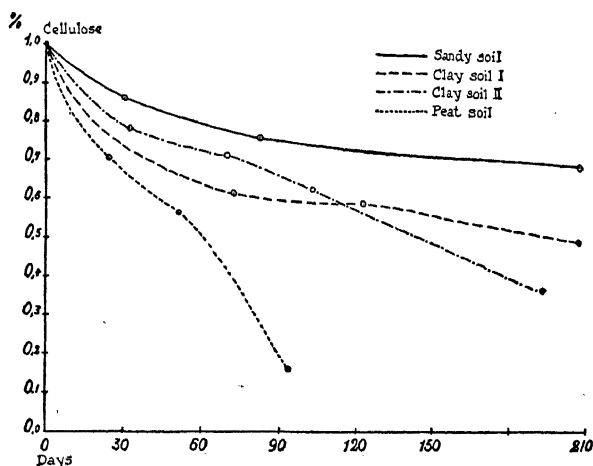


FIG. 17. Influence of soil type upon the decomposition of cellulose (after Charpentier).

However, the decomposition of the organic matter of the soil itself, such as the humus of mineral soils or peat and other decomposed or semi-decomposed organic materials may be greatly stimulated by the addition of lime. Using  $\text{CO}_2$  evolution as an index of decomposition of organic matter, Potter and Snyder<sup>60</sup> found that  $\text{CaCO}_3$  accelerates the rate of decomposition of the organic matter present in the soil or added in the form of stable manure (10 to 50 tons per acre).

<sup>59</sup> Jensen, H. L. Jour. Agr. Sci., 21: 81–100. 1931.

<sup>60</sup> Potter, R. S. and Snyder, R. S. Iowa Agr. Exp. Sta. Res. Bul. 39: 255–309. 1917; see also Lemmermann, O., Aso, K. Fischer, H. and Fresenius, L. Landw. Jahrb., 41: 217–256. 1911.

To stimulate cellulose decomposition in the soil, it may be sufficient to add the proper nutrients, such as phosphoric acid or nitrogen substances. According to Charpentier<sup>61</sup> the addition of 2 per cent of cow or horse manure greatly stimulates cellulose-decomposition in soil. The influence of the manure depends on the content of nutrients in the manure and in the soil. With a sufficient amount of moisture and a favorable reaction, the action of manure is greater with a higher content of nutrients in the manure and a lower content in the soil. The greater the moisture content of the soil, the quicker is the cellulose decomposed. Cellulose decomposition is active both in acid and alkaline soils, lime playing the part of a regulator of soil reaction. The addition of equal amounts of nitrogen in the form of ammonium sulfate and cow

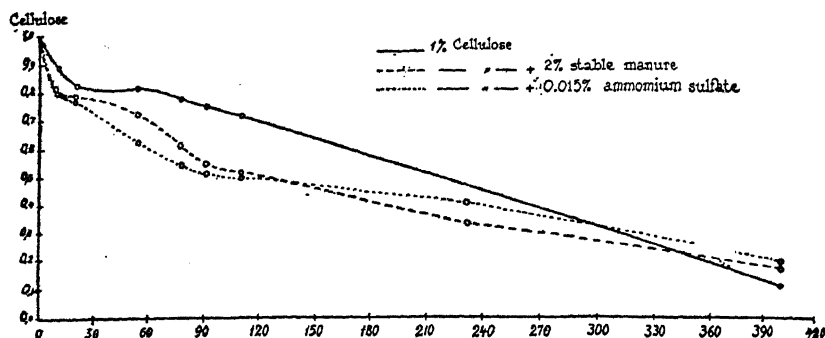


FIG. 18. Influence of nitrogen source upon the decomposition of cellulose in the soil (after Charpentier).

manure brings about an equal stimulation of cellulose decomposition in the soil. This points conclusively to the fact that the stimulating influence of cow and horse manure is due to its nitrogen content. Tables 32 and 33, and figures 17 and 18 show the influence of manure, soil reaction and nitrogen source on cellulose decomposition in various soils, as determined by Charpentier.

The content of available nitrogen in the soil is thus found to be the most important factor controlling cellulose decomposition. The ratio between the cellulose decomposed and nitrogen required by the organism is about 30-35 to 1.<sup>62</sup> This ratio depends of course upon the amount

<sup>61</sup> Charpentier, 1921 (p. 375).

<sup>62</sup> Heukelekian, O. and Waksman, S. A. Jour. Biol. Chem., 66: 323-342. 1925; Anderson, J. A. Soil Sci., 21: 115-126. 1926.

of available nitrogen. When the latter is in excess and is, therefore, not the limiting factor, the above ratio holds true. When the amount of available nitrogen is low, it will be utilized by the organisms over and over again, i.e., a part of the synthesized protoplasm of the micro-organisms will be decomposed, liberating some of the nitrogen which is immediately again assimilated thus enabling the organisms to decompose more cellulose. The process will be continuous, tending to give a higher ratio between the cellulose decomposition and the apparent nitrogen assimilation.

TABLE 32

*Composition of soils used for study of cellulose decomposition*

SOIL TYPE	DRY MATTER PER CENT	ASH PER CENT OF DRY MATTER	CELLULOSE PER CENT OF DRY MATTER	pH
Clay soil I.....	82.0	94.8	0.06	6.12
Clay soil II.....	82.0	93.5	0.01	7.08
Sandy soil.....	92.0	97.8	0.04	6.56
Peat soil.....	40.0	19.9	0.03	5.44

TABLE 33

*Influence of cellulose (1 per cent) upon soil reaction (using clay soil I)*

	NO LIME		WITH LIME (0.5 PER CENT CaCO <sub>3</sub> )	
	pH at beginning	pH at end	pH at beginning	pH at end
Unmanured soil.....	6.12	5.54	7.73	7.37
2 per cent cow manure.....	6.12	6.30	7.73	7.77

*Chemistry of hemicelluloses.* Hemicelluloses are amorphous polysaccharides, which are distinguished from cellulose by their easy solubility in dilute alkalies and in hot dilute acids, such as 1 per cent HCl. They are hydrolyzed readily by hot dilute mineral acids, but not by diastatic enzymes, and are thus distinguished from starch. Some give the brown to black iodine reaction like cellulose. On hydrolysis, hemicelluloses give glucose, mannose, galactose, levulose, or mixtures of these, as well as xylose and arabinose; hemicelluloses are thus termed dextrans, mannans, galactans, levulans, manno-galactans, pentosans (xylans, arabans), according to the constituent monosaccharides. Fre-

quently hemicelluloses consist of sugars linked with uronic acids,<sup>63</sup> largely glucuronic and galacturonic. Here belong the pectins and the gums, including the slimy substances synthesized by bacteria.

Hemicelluloses are present not only in higher plants, but also in algae, fungi, mosses,<sup>64</sup> and lichens (lichenin). Cellulose is commonly believed to serve as a protective substance in plants and is unaffected by plant metabolism, while hemicelluloses are reserve materials which must be brought into a soluble form by means of enzymes, before they can be utilized in plant nutrition; in some plants they serve for structural purposes holding the fibers together. The hemicelluloses thus comprise two different groups of substances: (1) the reserve hemicelluloses (mostly mannans) of seed, and to some extent of grasses; (2) supporting substances, mostly galactans and pentosans, having a mechanical function. The reserve hemicelluloses and starches can take the place of one another, so that seeds poor in starch are rich in reserve hemicelluloses and vice versa.

Figures usually quoted for the total furfuraldehyde yield of plant substances are unreliable because of the fact that the uronic acid groups ( $C_6H_{10}O_7$ ) of the hemicelluloses are hydrolyzed on boiling with acid into pentose ( $C_5H_{10}O_5$ ) and carbon dioxide ( $CO_2$ ). We come to recognize more and more that very few hemicelluloses are present in a pure state in plants. Cereals, wood and other plant materials contain several hemicelluloses. Oat straw contains, according to Norman, 22.8 per cent hemicellulose, which can be divided into an A and B preparation. A consists of about 11 per cent uronic acid anhydride, 79 per cent arabinose and xylose and 10 per cent anhydro-galactose. B yields about the same per cent of furfuraldehyde, but contains 32 per cent uronic acid anhydride and 68 per cent arabinose. Rye straw contains 33 per cent hemicellulose, A consisting of 5 per cent uronic acid anhydride, 60 per cent anhydropentose and 35 per cent anhydrohexose, and B containing 29 per cent uronic acid anhydride, 60 per cent anhydropentose and 11 per cent anhydrohexose. Most of our information on the hemicelluloses in plants and in soils is limited to the pentosans. A large part of these pentosans are probably derived from uronic acids. This information will have to be modified as our knowledge concerning these complexes increases.

<sup>63</sup> Norman, A. G. *Biochem. Jour.*, **23**: 1353-1366, 1367-1384. 1929; O'Dwyer. *Biochem. Jour.*, **17**: 501. 1923; **20**: 656. 1926; Candlin and Schryver. *Proc. Roy. Soc. London. B*, **103**: 365. 1928; Norris, F. W. and Preece, I. A. *Biochem. Jour.*, **24**: 59-66. 1930.

<sup>64</sup> Swartz, M. D. *Trans. Conn. Acad. Arts Sci.*, **16**: 247-382. 1911.

Pentosans are present in the cell walls of all green plants, in the bark and woody fiber of trees, in mosses, fungi, seeds and fruits. They occur very abundantly in straw which usually contains 23 to 29 per cent pentosan. Corn cobs contain 32 per cent pentosan, pine needles 6.8 per cent, oak leaves 10.3 per cent. Older tissues contain larger quantities of pentosans than younger ones. The pentosan content of the corn plant, for example, increases from 7.4 per cent in the kernel to 31.8 per cent in the cob at maturity.<sup>65</sup> Pentosans are of importance in the formation of wood and skeletal structure of various plants. The formula for pentosans is  $(C_5H_8O_4)_n$ , yielding, on hydrolysis,  $C_5H_{10}O_5$  or pentoses. The quantitative method of determining pentosan is based on the formation of a pentose from the pentosan, and of furfural from the pentose on further heating with acid. Pentosans are usually determined quantitatively by the phloroglucinol method.<sup>66</sup>

Humus soils are rich in pentosans or substances which yield furfural on boiling with HCl.<sup>67</sup> Forest soil, with 23.42 per cent humus, was found to contain 0.75 per cent pentosan; garden soil, with 9.85 per cent humus, contained 0.39 per cent pentosan; sandy soil, with 2.68 humus, contained only 0.04 per cent pentosan. Shorey and Lathrop<sup>68</sup> found pentosans universally distributed in the soil, the pentosan carbon ranging from 1.3 to 28.53 per cent of the total carbon. As much as 2.75 per cent pentosan was found in a North Dakota soil in which flax had been grown for a number of years. Most of this pentosan is probably found in the soil not as pentosan but as uronic acid compounds, which also give furfuraldehyde on boiling with 12 per cent HCl.

Pectins, pectous substances and pectoses are present in the intracellular substance (middle lamella) of parenchymatous plants. These substances are insoluble in cold water. Heating for a few minutes in an acid solution transforms the insoluble pectoses into soluble pectins; on heating with an alkali, the latter are changed to pectic acid. According to Ehrlich,<sup>69a</sup> pectin is changed on heating with water to hydro-

<sup>65</sup> Ver Hulst, J. H., Peterson, W. H. and Fred, E. B. *Jour. Agr. Res.*, **23**: 655-663. 1923.

<sup>66</sup> Kröber, E. *Jour. Landw.*, **48**: 357-384. 1900; Powell, W. J. and Whittaker, H. *Jour. Soc. Chem. Ind. Trans.*, **43**: 35-36. 1924; Youngburg, G. E. and Pucher, G. W. *Jour. Biol. Chem.*, **61**: 741-746. 1924; See also Pervier, N. C. and Gortner, R. A. *Jour. Ind. Engin. Chem.*, **15**: 1167, 1255. 1923.

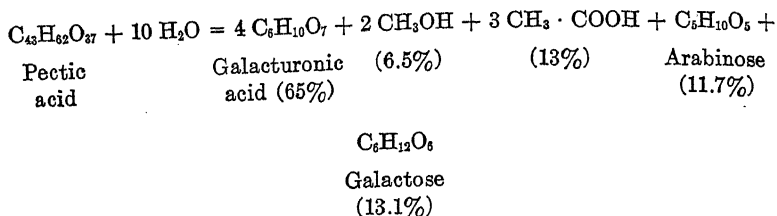
<sup>67</sup> Chalmot, G. de. *Amer. Chem. Jour.*, **16**: 218-223, 229. 1894.

<sup>68</sup> Shorey, E. C., and Lathrop, E. C. *Jour. Amer. Chem. Soc.*, **32**: 1680-1683. 1910; Shorey, E. C., and Martin, J. B., *Ibid.*, **52**: 4907-4915. 1930.

<sup>69a</sup> Ehrlich, F. *Ztschr. angew. Chem.*, **40**: 1307. 1927; **42**: 499. 1929; *Biochem. Ztschr.*, **212**: 162-239. 1929; *Ber. deut. chem. Gesell.*, **62**: 1974-2027. 1929; von Fellenberg, T. *Biochem. Ztschr.*, **85**: 82, 118. 1918.



pectin, a mixture of araban and Ca-Mg-pectate. The latter is hydrolyzed by acids and alkalis.



Pectins of various origin vary in the amount of galacturonic acid that they contain. This may be partly the reason for the different results obtained by different investigators. Microbial enzymes are capable of changing the insoluble pectin into insoluble hydropectin.

*Decomposition of hemicelluloses by microorganisms.* When plant substances undergo decomposition in soil or in compost, the hemicelluloses are at first attacked very rapidly, and then remain at a certain level while the cellulose continues to decompose. This is due to the non-homogenous character of the hemicelluloses, some decomposing very readily, more so than cellulose, and others being more resistant to decomposition than cellulose.

Various microorganisms are capable of decomposing hemicelluloses, including fungi, bacteria and actinomyces. The fungi include various species of *Aspergillus*, *Penicillium*, *Mucor*, *Rhizopus* and others.<sup>69</sup> Certain lower animals are also capable of assimilating hemicelluloses.<sup>70</sup> The hydrolysis of these polysaccharides is carried on by means of an enzyme *cytase*.<sup>71</sup>

Schmidt, Peterson and Fred<sup>72</sup> tested the pentosan decomposing power of a number of different fungi, by adding 1 gram of corn stover or rye straw to 25 cc. of a nutrient mineral solution containing  $\text{NH}_4\text{NO}_3$  as the only source of nitrogen. Different organisms were found to vary in their ability to decompose pentosans. It is interesting to note that a suspension of soil or of cow feces, containing a mixed flora, did not decompose any more pentosan than pure cultures of fungi. Wood

<sup>69</sup> Hérissé, H. Thèse. Paris. 1903; Schellenberg, 1908 (p. 247); Otto, 1916 (p. 248); Waksman, S. A. and Diehm, R. A. *Soil Sci.*, **32**: 97-118. 1931.

<sup>70</sup> Bierry, H. and Giaja, J. *Biochem. Ztschr.*, **40**: 370. 1912.

<sup>71</sup> Newcombe, F. C. *Ann. Bot.*, **13**: 49-81. 1899.

<sup>72</sup> Schmidt, E. G., Peterson, W. H. and Fred, E. B. *Soil Sci.*, **15**: 479-488. 1923; Ver Hulst, Peterson and Fred, 1923 (p. 393).

pentosan (alder, poplar, birch) was not destroyed in solution, due to the presence of a substance inhibiting the growth of the fungi. When added to the soil, 37 to 72 per cent of the wood pentosan was decomposed. Pure cultures of bacteria were also found capable of decomposing pentosans in the corn, varying from 1.7 to 12.8 per cent. The maximum destruction of the pentosan (12.8 per cent) was made by *Bac. flavigena*, a cellulose decomposing organism. Among the other hemicelluloses decomposed by bacteria, we find also agar-agar.<sup>73</sup> Pringsheim<sup>74</sup> demon-

TABLE 34  
*Decomposition of pentosans by fungi and by a mixed flora*<sup>72</sup>

ORGANISM	CORN STOVER PENTOSAN DESTROYED IN 142 DAYS	RYE STRAW PENTOSAN DESTROYED IN 300 DAYS
	<i>per cent</i>	<i>per cent</i>
<i>Asp. flavus</i> .....	40.1	38.1
<i>Asp. fumigatus</i> .....	53.0	35.1
<i>Asp. niger</i> .....	44.5	33.9
<i>Asp. oryzae</i> .....	38.0	
<i>Asp. repens</i> .....	31.0	
<i>Pen. glaucum</i> .....	33.0	36.6
<i>Cunninghamella</i> sp.....	42.5	
<i>Rhizopus nigricans</i> .....	29.5	
Soil suspension.....		35.1
Cow feces.....		33.2

strated that a trisaccharide is formed as an intermediary product of the decomposition of mannans by bacteria.

Various intestinal bacteria decompose xylan with the formation of large quantities of fatty acids, consisting of about 8 parts of acetic to 1 part of butyric acid.<sup>75</sup> According to Swartz,<sup>76</sup> various soil bacteria, especially certain anaerobic forms, are capable of decomposing pentosans, mannans and levulans; galactans are more resistant.

<sup>72</sup> Gran, H. H. Bergens Mus. Aarborg, 1902, H. I. Biernacki, W. Centrbl. Bakt. II, 29: 166-169. 1911. Gray and Chalmers, 1925 (p. 193); Lundestad, J. Centrbl. Bakt. II, 75: 321-344. 1928; Aoi, K. and Orikura, J. Ibid., 74: 321-333. 1928.

<sup>74</sup> Pringsheim, H. Ztschr. physiol. Chem., 80: 376-382. 1912; see also Cramer. Inaug. Diss. Halle. 1910.

<sup>75</sup> Seillière, G. Compt. Rend. Soc. Biol., 68: 991. 1910.

<sup>76</sup> Swartz, 1911 (p. 392); Waksman, S. A. and Diehm, R. A. Soil Sci. 32: 119-140. 1931.

Pectins are readily decomposed by various aerobic and anaerobic bacteria,<sup>77</sup> and fungi. Sugars are claimed to be the primary products formed from the hydrolysis of pectins. Among the secondary products, we find volatile acids (acetic and butyric), hydrogen and carbon dioxide. Some organisms produce formic, lactic and succinic acids, in addition to CO<sub>2</sub>, H<sub>2</sub> and acetic acid. In the process of retting of flax and hemp, especially under anaerobic conditions, alcohols and acetone are also formed, in addition to the above products.

Different species of fungi, like *Rhizopus*, both parasitic and non-parasitic forms, secrete an enzyme pectinase which dissolves the middle lamella of potatoes and of other plants.<sup>78</sup> A similar enzyme is formed by *Bac. carotovorus* in the rotting of carrots.<sup>79</sup> Microorganisms capable of decomposing pectins do not usually possess the ability of attacking cellulose, otherwise the retting process would be accompanied by the destruction of the cellulose of the fibers. Certain levulosans are not readily acted upon by microorganisms.<sup>80</sup>

*Lignins and their decomposition.* Lignin, or the non-carbohydrate portion of the lignified tissues, after it has been freed from fats, waxes, resins and tannins, is, next to cellulose and hemicelluloses, the most abundant constituent of plant tissues. Sphagnum moss contains 9 to 13 per cent lignin, cereal straw 18 to 22 per cent, saw grass, reeds and wood 28 to 37 per cent, and nut shells up to 47 per cent. In the plant tissues, lignin is present in a free state only to a very inconsiderable extent, but largely in the form of compounds with cellulose. Ligno-celluloses have a higher carbon content (47 to 50 per cent) than pure cellulose, due to the presence of the lignin, which contains 62 to 64 per cent carbon. The exact chemical nature of the lignin itself is still a matter of dispute.

The cell wall of plants consists of practically pure cellulose in the early stages of growth, but this is changed into ligno-cellulose with advancing growth; the lignin is formed from carbohydrates originally present in the cell wall. It is made up of hydrosols of high molecular weight which are adsorbed from the sap by the cellulose fibers.<sup>81</sup> Maximum lignification corresponds with maximum percentage of adsorbed

<sup>77</sup> Rossi, G. Intern. Rev. Sci. Pract. Agr., 7: No. 8. 1916; Norman, A. G. Ann. Bot., 43: 233-243. 1929; Pitman, G. A. and Cruess, W. V. Jour. Ind. Eng. Chem., 21: 1292-1295. 1929.

<sup>78</sup> Harter, L. L. and Weimer, J. L. Jour. Agr. Res., 22: 371-377. 1921; Amer. Jour. Bot., 10: 127-132, 167-169. 1923.

<sup>79</sup> Jones, 1909 (p. 198).

<sup>80</sup> Colin, H. and Estienne, V. Bull. Soc. Chim. Biol., 6: 431-435. 1924.

<sup>81</sup> Esselen, G. J. Jour. Ind. Engin. Chem., 15: 306-307. 1923.

material; this may be followed by chemical reactions, such as dehydration. The lignin content of rye straw was found<sup>82</sup> to increase with age, the greatest increase occurring during the period preceding ear formation. In the ligno-cellulose complex, the cellulose is believed to be linked with two non-cellulose substances one of which contains an aromatic nucleus, while the other is presumed to be a pentosan since it yields furfural on distillation with HCl.

Two different processes are available for the preparation of lignin, based upon the fact that it is insoluble in concentrated acids and is soluble in alkalis, when heated under pressure; acid or alkali lignin are thus obtained. To prepare acid lignin, finely ground straw or wood is extracted with ether and then treated with a concentrated acid, using either 72 per cent  $\text{H}_2\text{SO}_4$ , or fuming HCl solution (specific gravity 1.21), or a mixture of 1 vol. HCl, specific gravity 1.07, and 6 vol. 72 per cent  $\text{H}_2\text{SO}_4$ .<sup>83</sup>

Alkali lignin can be prepared by extracting the plant material with NaOH solution. The actual amount of lignin thus obtained depends on the concentration of the alkali used, temperature and period of extraction. The following method was recommended:<sup>84</sup> Ten parts of a 10 per cent NaOH solution are added to one part of finely ground straw, wood, or soil containing these materials, and the mixture is heated at  $130^\circ$  under pressure, for one hour or more. Two volumes of water are then added to the digested mixture and the dark-colored solution containing the lignin is filtered off. The warm filtrate is acidified with hot hydrochloric acid, brought to boiling and the precipitated lignin is centrifuged or filtered off. To obtain pure lignin, this preparation is washed with hot dilute hydrochloric acid, dried, redissolved in a mixture of acetone and water, and reprecipitated by pouring into a mixture of hot hydrochloric acid (20 per cent) solution. The lignin is now filtered off, washed with hot water and dried at  $40^\circ\text{C}$ . The yield of lignin by alkali extraction is considerably lower than that obtained by treatment

<sup>82</sup> Beckmann, E., Liesche, D. and Lehmann, F. *Ztschr. Angew. Bot.*, **34**: 285-288. 1921; *Biochem. Ztschr.*, **139**: 491-508. 1923.

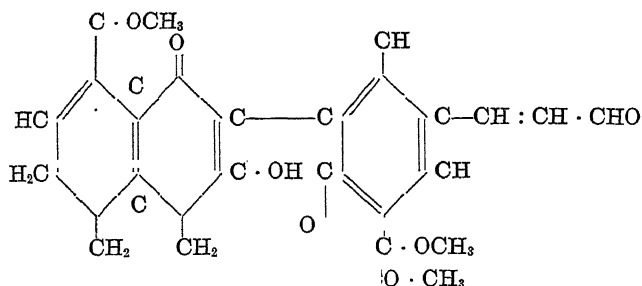
<sup>83</sup> Willstätter, R. and Zeichmeister, L. *Ber. deut. chem. Gesell.*, **46**: 2401-2412. 1913; Hägglund, E. and Bjorkman, C. B. *Biochem. Ztschr.*, **147**: 74-80. 1924; *Cellulosechemie*, **4**: 74-77. 1923; Schwalbe, H. *Papierfabr.*, **23**: 174-177. 1925; Wenzl, H. *Papierfabr.*, **23**: 305-306. 1925; Schorger, 1926 (p. XVIII); Fuchs, W. *Die Chemie der Lignine*. Berlin. 1926.

<sup>84</sup> Powell, W. J. and Whittaker, H. *Jour. Chem. Soc. Trans.*, **125**: 35-36. 1924; see Phillips, M. *Jour. Amer. Chem. Soc.*, **51**: 2420-2426. 1929.

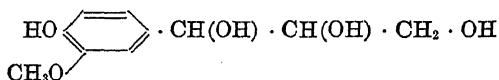
with strong acids. By raising the temperature of extraction to  $180^{\circ}\text{C}$ ., a yield almost equivalent to that of acid lignin may be obtained.<sup>85</sup>

It is doubtful whether lignin is a single chemical compound, Ritter<sup>86</sup> having shown that it can be separated even by mechanical means into two forms, one of which is located in the middle lamella of the tree and has a methoxyl content of 10.8 to 13.6 per cent and the other is cell wall lignin with a methoxyl content of 4.3 to 4.8 per cent. By treatment with alcohol or with  $\beta$  naphthol, lignin can also be separated into two fractions, one soluble and the other insoluble. A close relationship was found to exist between certain alcohol-soluble resins, tannins, and lignin.

Various formulae have been suggested to account for the chemical composition of lignin. It is sufficient to give the  $\alpha$ -lignin formula of Klason.<sup>87</sup>



Klason believed that lignin consists of 6 groups, each containing 2 molecules of coniferyl aldehyde and 1 molecule of water, or  $(2\text{C}_{10}\text{H}_{10}\text{O}_3 \cdot \text{H}_2\text{O})_6$ . According to this formula, the methoxyl content of lignin is 16.5 per cent. Freudenberg,<sup>88</sup> however, suggested for lignin the formula of an anhydride of  $\alpha$ -vanillyl-glycerol:



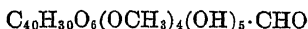
<sup>85</sup> Mehta, M. M. *Biochem. Jour.*, **19**: 958-978. 1925.

<sup>86</sup> Ritter, G. J. *Jour. Ind. Engin. Chem.*, **17**: 1194-1197. 1925.

<sup>87</sup> Klason. *Ber.*, **55**: 448-455. 1922; see Rassow, B. and Zickmann, P. *Jour. prakt. chem.*, N. F. **123**: 189-234. 1929; Kürschner, K. *Technol. Chem. Pap. Zellst. Fahr.*, **26**: 53-66. 1929.

<sup>88</sup> Further information on the chemistry of lignins is found in the book of W. Fuchs, *Die Chemie des Lignins*. Berlin. 1926; Kürschner, K. *Festschr. J. Stoklasa*, 219-245. 1928; Freudenberg, K. et al. *Ber. deut. chem. Gesell.*, **62**: 1554, 1814. 1929; **63**: 792-795. 1930.

Lignin is made up of twelve groups of this compound with ether-like linkages and with a loss of 7 molecules of water ( $C_{120}H_{132}O_{42}$ ). Powell and Whittaker suggested the following formula for flax lignin:



and for rye lignin:  $C_{40}H_{44}O_{15}$ . The carbon content of lignin was found to be about 63.0 per cent.

The methods of determination of lignin in plant organic substances are based either upon the destruction of all the carbohydrates with concentrated acids, or upon the oxidation of lignin with chlorine dioxide or other oxidizing agent, or upon the determination of some chemical constituent of lignin, such as the methoxyl groups.<sup>89</sup> However, none of these methods are very accurate. Alkalies extract, under pressure, only a part of the lignin; but this method can be applied most readily to soils.

For the study of its decomposition by microorganisms, lignin can be added, either in crude or pure form, to soil or to a solution containing a source of nitrogen and the necessary minerals. The growth of the organisms, the evolution of  $CO_2$ , or the disappearance of the lignin added can be taken as quantitative indices of decomposition. Pringsheim and Fuchs<sup>90</sup> dissolved 10 grams of lignin (obtained by treating spruce wood with 11.5 per cent NaOH under pressure, then precipitating the lignin with HCl and heating in the presence of an excess of 2 per cent acid) in  $NH_4OH$  and then warmed the solution to drive off the excess of ammonia. The solution thus obtained was added to 5 liters of a nutrient medium containing 20 grams  $(NH_4)_2SO_4$ , 3 grams  $K_2HPO_4$ , 2.5 grams  $MgSO_4$  and 2 grams  $CaCO_3$ . The medium was inoculated with soil and incubated at  $37^\circ$ ; decomposition was found to take place. It resulted in the complete disappearance of the pentosan content and in the reduction of the methoxyl content of the lignin. The ability of certain bacteria to decompose lignins has also been pointed out by Schrader.<sup>91</sup> Attention must be called here to the fact that alkali lignin usually contains some pentosans and probably some proteins; it is these substances which frequently undergo decomposition and not the lignin

<sup>89</sup> Schorger, 1926 (p. XVIII); Schwalbe, C. G., Papier. Ztg. 13. 1920; see Rassow, B. and Gabriel, H. Cellulosechem. 12: 227-235. 1931.

<sup>90</sup> Pringsheim, H. and Fuchs, W. Ber. deut. chem. Gesell., 56: 2095-2097. 1923.

<sup>91</sup> Schrader, H. Ges. Abh. Chem. der Kohle., 5: 553; Chem. Centrbl., 4: 1044. 1922; 3-4: 1649. 1923.

itself, the latter being very resistant to the action of microorganisms.<sup>92</sup> Very few organisms are known to be capable of attacking lignins and use them as sources of energy.

Cellulose and other carbohydrate constituents of straw, such as the pentosans, are readily digested by animals, while the lignins are attacked only to a very limited extent; the methoxyl content is reduced, due probably to the action of enzymes in the digestive juice.<sup>93</sup> The presence of lignins which thoroughly impregnate the cellulose make the process of digestion even more difficult. When the lignins are made soluble or removed by alkaline treatment, the straw becomes a more available source of energy and its digestibility is greatly increased. This removal of the lignin can be accomplished by treating the straw with various concentrations of NaOH or Ca(OH)<sub>2</sub> at different temperatures and pressures. In the soil the cellulose and hemicelluloses are decomposed by microorganisms long before the lignin is appreciably acted upon.

In addition to possibly certain actinomyces and bacteria, higher fungi are capable of decomposing lignin to a limited extent, much less so than cellulose, and it is these organisms which are concerned largely with the rotting of wood. The mycelial filaments penetrate into the woody tissues and cause their decomposition. Most of these fungi belong to the Basidiomycetes, chiefly Hymenomycetes.<sup>94</sup> Czapek<sup>95</sup> suggested that two enzymes (hydromase and cellulase) are active in the reaction. Certain filamentous fungi seem to exert some action upon ligno-cellulose, as shown by Otto,<sup>96</sup> who found that *Trichothecium*, *Aspergillus* and *Mucor* can dissolve out certain substances from the organic complexes. The course of decomposition is as follows: The fungi first assimilate the sugars, then the dextrins and some of the hemicelluloses, and finally the cellulose is acted upon. The lignin and certain hemicelluloses are most resistant. The formation of humus in soil, especially in forest soils, is in close connection with the decomposition of wood; the organic substances are first attacked by fungi; a mixed flora of bac-

<sup>92</sup> Fischer, 1923 (p. 654); Waksman, 1928 (p. 589).

<sup>93</sup> Rubner, M. Sitz. preusz. Akad. wissenschaft., **12**: 127. 1928; Dore, W. H. and Miller, R. C. Univ. Cal. Publ. Zool., **22**: 383-400. 1923; Csonka, F. A., Phillips, M. and Jones, D. B. Jour. Biol. Chem., **85**: 65-76. 1929.

<sup>94</sup> Rudan, B. Beitr. Biol. Pflanz., **13**: 375. 1917; Hubert, E. E. Jour. Agr. Res., **29**: 526. 1924.

<sup>95</sup> Czapek, F. Ber. deut. bot. Gesell. 1899; Ztschr. physiol. Chem., **27**: 141-166. 1899.

<sup>96</sup> Otto, 1916 (p. 248); Ward, H. M. Ann. Bot., **12**: 565. 1898; Schellenberg, H. G. Centrbl. Bakt. II, **55**: 351. 1922.

teria and fungi then follows, finally a rich fauna of lower animals. Among the residual substances, which go to form the so-called humus and humic acids, lignin occupies a leading place.

The ability of decomposing lignins has been established with certainty only for very few fungi, all belonging to the Hymenomycetes. Falck<sup>97</sup> distinguishes between *corrosion*, whereby the lignins are decomposed completely and the celluloses only to a limited extent, this process

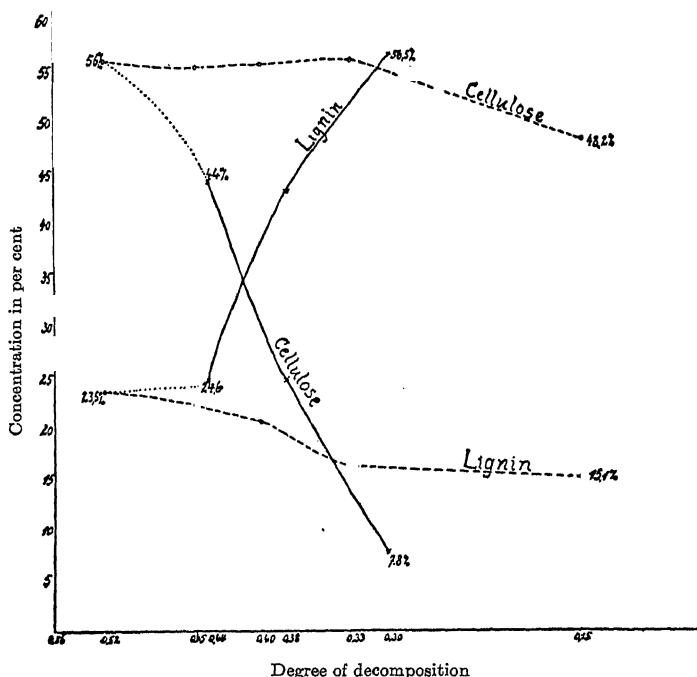


FIG. 19. Degree of decomposition of lignin and cellulose in the processes of destruction and corrosion brought about by microorganisms decomposing wood (from Falck). x———x destruction; o-----o corrosion.

being carried out by wood parasitic fungi (*Polyporus annosus* and *Trametes pini*), and *destruction*, whereby only the celluloses are decomposed and not the lignins, the residue falling apart as dust; the latter process is carried out by *Merulius*, *Coniophora*, *Poria*, *Lenzites* and other fungi. In destruction, the residual material is dark and readily dissolves in alkalis. Pentosans are attacked in both cases. Destruction may

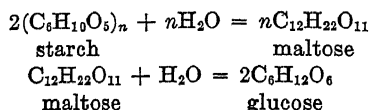
<sup>97</sup> Falck, R. Ber. deut. bot. Gesell., 44: 652-664. 1927; Ber. deut. Chem. Gesell., 60: 225-232. 1927.



follow corrosion, but not vice versa. Destruction leads to humus formation (so-called raw humus), with an increase in the carbon content of the residual material.<sup>98</sup> This is brought out in Fig. 19.

It may be added here that, as far as our present information is concerned, corky and cutinized lamellae are not acted upon to any extent by microorganisms.<sup>99</sup>

*Starches and their decomposition by microorganisms.* Starches are predominantly reserve materials, forming 60 to 70 per cent of the dry weight of cereal grain. They are complex carbohydrates of the formula  $(C_6H_{10}O_5)_n \cdot H_2O$ , but are more readily soluble than cellulose and give the characteristic blue color with iodine. They swell in hot water. They are much more readily acted upon by microorganisms than cellulose and hemicelluloses, due to the fact that a large number of soil bacteria, actinomyces and fungi produce very active diastatic enzymes which hydrolyze the starches first into dextrins of different complexity, then into sugars:



The ability of certain bacteria and fungi to produce enzymes hydrolyzing starch is so great that the processes have been utilized for various commercial purposes where diastatic enzymes are required. Certain bacteria, however, are capable of breaking down starches with the formation of acids, alcohols and acetone.<sup>100</sup> In this, the action of bacteria upon starch may be distinct from that of diastatic enzymes, which give 100 per cent maltose, and from the acid hydrolysis of starch, which results in the formation of glucose.

The number of organisms in the soil capable of hydrolyzing starch can be readily determined. The soil is diluted 1:1000 to 1:200,000. The final dilution is plated out on a medium which consists of 15 grams potato starch, 1 gram of an organic or inorganic source of nitrogen, 0.5 gram  $K_2HPO_4$ , 15 grams of agar and traces of  $MgSO_4$  and  $FeCl_3$  in 1000 cc. of water. After a few days incubation (3 to 7), the plates are covered with a dilute solution of iodine and potassium iodide. The

<sup>98</sup> Further information on the transformation of lignins in the decomposition of plant materials is given elsewhere (p. 680).

<sup>99</sup> Miyoshi, M. *Jahrb. Wiss. Bot.*, **28**: 269-289. 1895; Otto, 1916 (p. 248).

<sup>100</sup> Schardinger, F. *Centrbl. Bakt.* II, **14**: 772-781. 1905; **19**: 161-163. 1907; **22**: 98-103. 1909; 188-197. 1911.

colonies of the microorganisms producing diastase will be surrounded with a clear zone; these colonies may then be counted. Numerous bacteria are capable of decomposing starch, including various spore forming organisms, such as *Bac. subtilis*, *Bac. mesentericus*, *Bac. cereus* and other common aerobic soil bacteria, and also various anaerobic organisms, including butyric acid bacteria. Certain non-spore bearing bacteria, such as various cellulose-decomposing organisms, are also capable of decomposing starch. The ability to hydrolyze starch is widely distributed among the fungi, such as *Asp. oryzae*, *Asp. niger* and *Amylomyces boidin*.

Formic, acetic and butyric acids, traces of lactic and succinic acids, various alcohols (ethyl and butyl), aldehydes and acetone, hydrogen and carbon dioxide have been obtained among the products of decom-

TABLE 35

*Influence of age of culture upon the fat content of Asp. niger*<sup>101</sup>  
350 cc. Raulin's solution containing 4.7 per cent invert sugar

AGE OF CULTURE	RESIDUAL SUGAR	DRY WEIGHT OF MYCELIUM	FAT CONTENT
<i>days</i>	<i>gm.</i>	<i>mgm.</i>	<i>per cent</i>
1	8.6	0.29	2.11
2	1.9	5.10	12.0
3	0	6.30	7.5
4	0	4.1	4.0
7	0	1.7	1.6
12	0	1.3	0.6

position of starches by microorganisms. *Bac. mesentericus*, for example, breaks down starches into carbon dioxide, formic and valerianic acids. *Bac. granulobacter pectinovorum* growing in media rich in starch produces acetic and butyric acids, a part of which is reduced to the corresponding alcohols.<sup>102</sup>

Inulin, similar in its properties to starch, but giving levulose on hydrolysis, can also be decomposed by various bacteria and fungi.<sup>103</sup>

*Decomposition of fats and waxes.* Fats are widely distributed in the plant and animal residues added to the soil. They are also synthesized by the different groups of soil microorganisms. The amount of

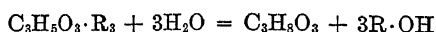
<sup>101</sup> Dubaquié. Mem. Soc. Sci. Phys. Nat. Bordeaux (6). 1910; Terroine, E. F. and Bonnet, R. Bull. Soc. Chim. Biol., 9: 588-596. 1927.

<sup>102</sup> Speakman, H. B. Jour. Biol. Chem., 41: 319-343. 1920.

<sup>103</sup> Grafe, V. and Vouk, V. Ztschr. Gärungsphys., 3: 327-333. 1913; Kiesel, A. Ann. Inst. Past., 28: 747-757. 1914.

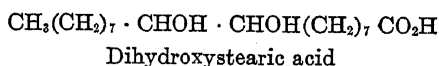
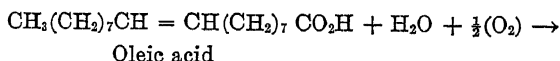
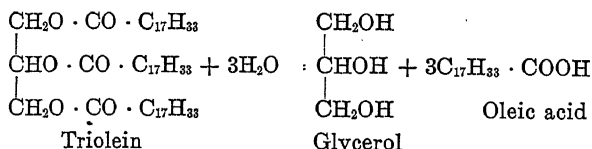
fat synthesized and the nature of the fat will depend upon the type of organism and stage of growth (Table 35). The fat is a reserve substance and is readily utilized by the organisms in the absence of other available sources of energy.<sup>101</sup>

Fats decompose only slowly in moist soils, and almost not at all in dry soils. According to Rubner,<sup>104</sup> only 22.9 per cent of butterfat added to soil (4.5 grams of fat to 200 grams of soil) was decomposed during a period of one year and 38.1 per cent in twelve years; other fats were decomposed at a different rate. The fats are first hydrolyzed, according to the general reaction:



The glycerol or corresponding alcohols are readily utilized by various groups of microorganisms as sources of energy, while the fatty acids are decomposed further. The ease with which unsaturated fatty acids are decomposed by bacteria and fungi depends upon the number of double bonds in the fatty acid molecule and not upon the physical properties of the latter. The utilization of palmitic and of stearic acids is probably preceded by a spontaneous dehydrogenation of the acids, which renders them more available.<sup>105</sup>

A typical fat is decomposed in the soil as follows:



This last substance has been demonstrated in the soil<sup>106</sup> and was also found in the cells of fungi. Fats are decomposed chiefly by fungi,

<sup>104</sup> Rubner, N. Arch. Hyg., 91: 290. 1922.

<sup>105</sup> Haag, F. E. Arch. Hyg., 100: 271-308. 1928; see also Flieg, O. Jahrb. Wiss. Bot., 61: 24-64. 1922; Coppock, P. D., Subrahmanyam, V. and Walker, T. K. Jour. Chem. Soc., 1928, 1422-1427; Terroine, E. F., Bonnet, R. and Duquenois, P. Bull. Soc. Chim. Biol., 9: 597-604. 1927.

<sup>106</sup> Schreiner, O. and Shorey, E. Bur. Soils, U. S. Dept. of Agr. Bul. 53. 1909.

with the possible formation of ketones, and by a number of aerobic bacteria, including *Staph. pyogenes aureus*, *Bact. prodigiosum*, *Bact. pyocyaneum*, *Bact. fluorescens*, *Bact. lipolyticum*.<sup>107</sup>

It is possible, however, that some fat may also be decomposed under anaerobic conditions. The chemical processes involved may be different, Bach and Sierp<sup>108</sup> having shown that, under anaerobic conditions, CO<sub>2</sub> is split off and the fatty acids change into hydrocarbons. This results in the formation of products of a lower saponification and higher iodine number than the original fat. Microorganisms are also capable of synthesizing fats, in varying amounts, depending upon the organisms and conditions of nutrition.

Waxes are chemically related to the fats, being esters of higher alcohols and fatty acids. For example, flax wax consists of phytosterol and ceryl alcohol, as well as of palmitic-, stearic-, oleic-, linolic-, and linoleic acids. These substances are even more resistant to decomposition than the fats; they are acted upon under aerobic conditions by soil fungi and certain bacteria.<sup>109</sup> It may be of interest to mention here that Greig-Smith<sup>110</sup> attempted to explain soil exhaustion as a result of an accumulation of fats and waxes ("agricere"); when these are partly removed by the action of volatile antiseptics, further decomposition of the soil organic matter sets in.

*Decomposition of paraffins, aliphatic hydrocarbons and benzene ring compounds in the soil.* According to Söhngen,<sup>111</sup> various non-spore forming bacteria and Mycobacteria are capable of oxidizing paraffin, benzin, petroleum and paraffin oil. On adding 2 grams of paraffin to the medium, incubating one month at 28°, then extracting the remaining paraffin with petroleum ether, it was found that the following amounts were decomposed:

<sup>107</sup> Schenker, R. *Biochem. Ztschr.*, **120**: 164-196. 1921; Derx, H. G. *Konigl. Akad. Wiss. Amsterdam*, **33**: 545-558. 1924; Söhngen, N. L. *Ibid.*, **19**: 698. 1910; **20**: 126. 1911; Eijkman, C. *Centrbl. Bakt. I*, **29**: 841-848. 1901; de Kruyff, E. *Bull. dept. agr. Ind. néerland. IX. Buitenzorg*. 1907. (*Centrbl. Bakt. II*, **20**: 610-611. 1908); Huss, H. *Centrbl. Bakt. II*, **20**: 474-484. 1908; Stephenson, M. and Whetham, M. D. *Proc. Roy. Soc. B.*, **93**: 262-280. 1922; Shibata, N. *Jour. Biochem. Tokyo*, **1**: 249-260. 1922; Zikes, H. *Centrbl. Bakt. II*, **69**: 161. 1926.

<sup>108</sup> Bach and Sierp. *Centrbl. Bakt. II*, **62**: 24-76. 1924. A detailed review of the formation and decomposition of fats by microorganisms is given by Seliber, G. in his monograph published by Glavnauka. Leningrad. 1926.

<sup>109</sup> Tausson, W. O. *Biochem. Ztschr.*, **193**: 85-93. 1928

<sup>110</sup> Greig-Smith, 1912 (p. 735).

<sup>111</sup> Söhngen, N. L. *Centrbl. Bakt. II*, **37**: 595-609. 1913; Wagner. *Ztschr. Gärungsphysiol.*, **1**: 289. 1914.

	mgm.
<i>Mycob. album</i> .....	300
<i>Mycob. rubrum</i> .....	330
<i>Micr. paraffinae</i> .....	180
<i>Bact. fluorescens liquefaciens</i> .....	180
Crude culture.....	540

In addition to the bacteria, certain fungi are also capable of utilizing paraffins as sources of energy. Tausson<sup>112</sup> found that *Asp. flavus* decomposed paraffin of a high melting point (+78°), with an economic coefficient of 53 to 66.5 per cent. Various other fungi and bacteria (*Bact. fluorescens*, *Bact. pyocyaneum*, *Bact. stutzeri*) are capable of using paraffins as well as kerosene as sources of energy.

Tausz and Peter<sup>113</sup> isolated from the soil 3 bacteria capable of decomposing paraffin hydrocarbons. *Bact. aliphaticum* decomposed quantitatively benzol, *n*-hexan ( $\text{CH}_3(\text{CH}_2)_4\text{CH}_3$ ), *n*-octan ( $\text{CH}_3(\text{CH}_2)_6\text{CH}_3$ ), di-iso-amyl ( $(\text{CH}_3)_2\text{CH}(\text{CH}_2)_4\cdot\text{CH}(\text{CH}_3)_2$ ), *n*-hexadecan ( $\text{CH}_3(\text{CH}_2)_{14}\text{CH}_3$ ), triocotan ( $\text{C}_{30}\text{H}_{62}$ ) and tetratriocotan ( $\text{C}_{34}\text{H}_{70}$ ), but not naphthenes. *Bact. aliphaticum liquefaciens* decomposed small quantities of naphthenes; the physical constants of the corresponding hydrocarbons change thereby giving higher values. *Paraffinobacterium* attacked the higher homologues of the paraffin series, from hexadecan. Cyclic hydrocarbons as benzol and cyclohexan were not attacked. The organisms could be used for testing the purity of naphthene hydrocarbons and for the separation of aliphatic hydrocarbons from naphthenes, since the former are decomposed quantitatively.

Tausz and Donath<sup>114</sup> later found that *Bact. aliphaticum liquefaciens* could oxidize hydrogen and the higher aliphatic hydrocarbons, beginning from pentane, while the methane-oxidizing organism could oxidize hydrogen and the lower as well as the higher aliphatic hydrocarbons. The ability of specific bacteria to attack certain hydrocarbons begins only with a certain member of the chain; if the lowest member of the homologue series is attacked by a certain organism, the higher members will also be attacked; the longer the chain, the easier it is attacked.

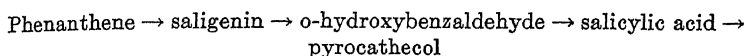
Benzene ring compounds, including phenol, cresol and naphthalene disappear rapidly in the soil, due largely to the action of various bacteria (mycobacteria, large, sporangia-producing rods, and short, oval pseudo-

<sup>112</sup> Tausson, W. O. Biochem. Ztschr., 155: 356-368. 1925; Neftyanoe Khozyaistvo, 14: 220-230. 1928; see also Rahn, O. Centrbl. Bakt., 16: 382-384. 1906.

<sup>113</sup> Tausz, O. and Peter, M. Centrbl. Bakt. II, 49: 497-554. 1920.

<sup>114</sup> Tausz, J. and Donath, P. Ztschr. physiol. Chem., 190: 141-168. 1930.

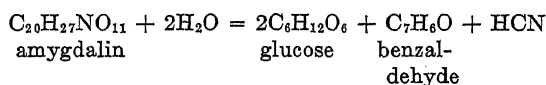
monads).<sup>115</sup> However, Sen Gupta<sup>116</sup> found that the disappearance of phenol in the soil is caused largely by the catalytic action of manganese oxide. Although naphthalene has a toxic action upon insects (wireworms) and bacteria, it is rapidly lost from the soil due to its decomposition by bacteria. Repeated additions of naphthalene leads to a more rapid decomposition. Determination of naphthalene in soil is best made by the use of picric acid, resulting in the formation of naphthalene picrate. As an illustration of the chemistry of decomposition of some of the benzene-ring compounds by bacteria, the transformation of phenanthrene may be cited.<sup>117</sup>



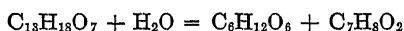
Aromatic compounds are much less favorable sources of energy for fungi, such as the Mucedinaceae, than aliphatic compounds.<sup>118</sup>

The latex hydrocarbons in rubber are decomposed by actinomyces and certain bacteria.<sup>119</sup>

*Decomposition of glucosides and monosaccharides.* Glucosides are widely distributed in the plant kingdom and are, therefore, introduced into the soil by various plant products. The first products of hydrolysis are glucose and other compounds, as shown in the case of amygdalin:



Salicin is decomposed into glucose and saligenin:



The hydrolysis of indican ( $\text{C}_{14}\text{H}_{17}\text{NO}_6$ ) with the formation of glucose and indoxyl ( $\text{C}_8\text{H}_6\text{NO}$ ), which changes in the air to indigo blue ( $\text{C}_{16}\text{H}_{10}\text{N}_2\text{O}_2$ ), belongs also to this type of reaction. The glucose is used by a great variety of microorganisms; benzaldehyde or the other benzol ring compounds as well as the hydrocyanic acid, are decomposed sooner or later.

<sup>115</sup> Thornton, H. Nature, 111: 347. 1923; Gray and Thornton, 1928 (p. 200).

<sup>116</sup> Sen Gupta, N. N. Jour. Agr. Sci., 11: 136-158. 1921; 15: 497-515. 1925.

<sup>117</sup> Tattersfield, F. Ann. Appl. Biol. 15: 57-80. 1920; Tausson, W. A. Ztschr. Wiss. Biol. Abt. 2. Planta., 4: 214-256. 1927; 5: 239-273. 1928; 7: 735-757. 1929.

<sup>118</sup> Coupin, H. *Compt. Rend. Acad. Sci.*, **185**: 145-146. 1927.

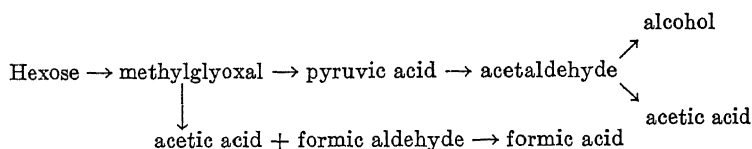
<sup>119</sup> Söhngen and Fol. Centrbl. Bakt. II, 40: 87. 1914; de Vries, O. Ibid., 74: 22-24. 1928.

Tannin is also used readily, especially in low concentrations, as a source of carbon by a number of microorganisms, particularly various fungi.<sup>120</sup>

Monosaccharides are acted upon by the great majority of heterotrophic microorganisms inhabiting the soil. The nature of the reaction depends upon the organism concerned and environmental conditions. Under certain conditions, the sugar is oxidized to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ , liberating the maximum amount of energy; under other conditions, acids, alcohols or both, with or without gases ( $\text{H}_2$ ,  $\text{CH}_4$ ,  $\text{CO}_2$ ), are formed. In addition to the organic acids produced by fungi<sup>121</sup> and bacteria, various alcohols, including ethyl-, methyl- and butyl-, and acetone, may also be produced especially by anaerobic bacteria. These substances are usually oxidized further or are resynthesized, with the result that complex products are formed again.

Just as gluconic acid in the animal body, gluconic acid is the first product in the decomposition of glucose by fungi. *Asp. niger* may produce both gluconic ( $\text{C}_6\text{H}_{12}\text{O}_7$ ) and citric acids ( $\text{C}_6\text{H}_8\text{O}_7$ ) from glucose, the latter being formed in more acid and the former in less acid media; neither is intermediary to the formation of the other; both are decomposed further to oxalic acid ( $\text{C}_2\text{H}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$ ). Out of 215 gm. of sugar decomposed in 1200 cc. of medium containing 0.15 per cent  $\text{NH}_4\text{NO}_3$ , there were formed 19 grams of fungus mycelium, 63.7 grams gluconic acid, 57.3 grams citric acid and 24.2 grams oxalic acid.<sup>122</sup>

According to Aubel,<sup>123</sup> hexoses are acted upon by *Bact. pyocyaneum* in the following manner:



*Bact. coli* changes glucose to ethyl alcohol, lactic and acetic acids,  $\text{CO}_2$  and  $\text{H}_2$ .



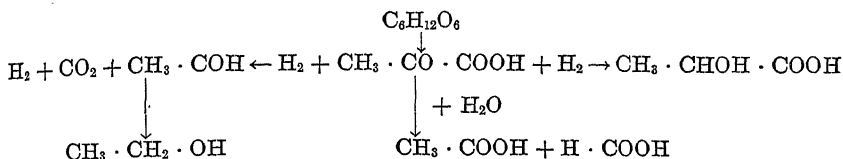
<sup>120</sup> Rippel, A. and Keseling, J. Arch. Mikrob., 1: 60-77. 1930.

<sup>121</sup> Butkewitch, V. S. and Fedorov, M. V. Biochem. Ztschr., 206: 440-456. 1929; 219: 87-102. 1930. Falck, R. and Kiuyma, B. Ber. deut. chem. Gesell., 57: 915-920, 920-923. 1924.

<sup>122</sup> Butkewitsch, W. Biochem. Ztschr., 154: 177-190. 1924; Jahrb. wiss. Bot., 64: 636-650. 1925; Bernhauer, K. Biochem. Ztschr., 153: 517-521. 1924.

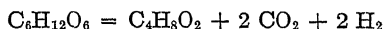
<sup>123</sup> Aubel, E. Compt. Rend. Acad. Sci., 175: 1493-1495. 1921.

As an instance of the chemistry of decomposition of starch or sugar under anaerobic conditions, it is sufficient to illustrate the action of *Bac. acetoethylicum*.<sup>124</sup>



The action of *Bac. granulobacter pectinovorum* upon starch, pentoses and hexoses results<sup>125</sup> in the formation of acetone, butyl alcohol, hydrogen and carbon dioxide, with various acids (butyric, acetic, lactic) as intermediary products. When aliphatic compounds with carboxyl groups are acted upon by *Bact. pyocyaneum*, they become alkaline as a result of the oxidation of the carboxyl groups. Compounds containing  $-\text{CHO}$  or  $-\text{OH} = \text{CO}-$  groups become acid as a result of oxidation.<sup>126</sup>

The formation of butyric acid by the various butyric acid bacteria under anaerobic conditions can be represented as follows:



This process is much more complex than represented by the above reaction, since other acids and various alcohols are also formed. The acid itself may be formed as a result of the synthetic processes rather than by direct decomposition.<sup>127</sup>

*Decomposition of organic acids.* Fatty acids are thus formed from poly- and mono-saccharides, from proteins and their derivatives. When neutralized, these acids serve as good sources of energy for various bacteria and fungi.<sup>128</sup> The great majority of heterotrophic bacteria can utilize malic, citric, fumaric, glyceric, succinic, formic, lactic, mucic, and tartaric acids; a small number utilize acetic, propionic, 'quinonic acids. Maleic,  $\beta$ -oxy-butyric,  $\alpha$ -oxy-butyric and oxalic acids are utilized only to a very limited extent. The decomposition of salts of these acids results in the formation of alkali carbonates, which lead to an alkaline reaction of the medium:

<sup>124</sup> Northrop, J. H., Ashe, L. H. and Senior, J. K. Jour. Biol. Chem., **39**: 1-21. 1919; Speakman, H. B. Jour. Biol. Chem., **64**: 41-52. 1921.

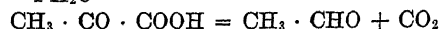
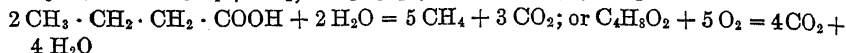
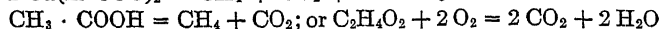
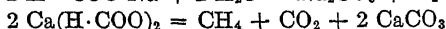
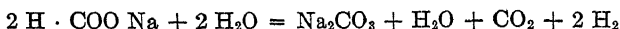
<sup>125</sup> Speakman, H. B. Jour. Biol. Chem., **58**: 395-413. 1923; Reilly, J. et al. Biochem. Jour., **14**: 229-251. 1920.

<sup>126</sup> Supniewski, J. Compt. Rend. Soc. Biol., **89**: 1377-1379. 1923.

<sup>127</sup> Neuberg, C. and Arinstein, B. Biochem. Ztschr., **117**: 269-314. 1921; Nord., F. F. Chem. Rev., **3**: 41-79. 1926.

<sup>128</sup> Coolhaas, C. **75**: 344-366. 1928.

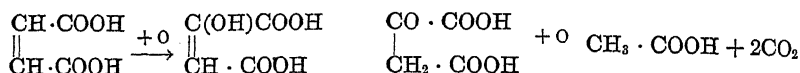




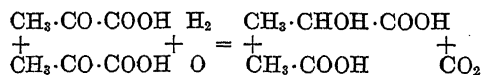
pyruvic acid

acetaldehyde

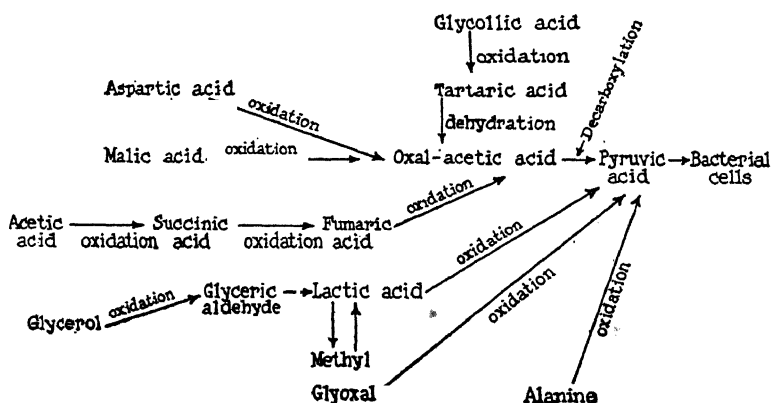
Fumaric acid is decomposed by *Bact. pyocyaneum*<sup>129</sup> to the lower fatty acids, chiefly acetic; pyruvic acid may also be isolated.



The rôle of pyruvic acid in fermentation processes (anaerobic utilization of energy) suggested by Neuberg and associates found support in various investigations on the nutrition of bacteria.<sup>130</sup>



The general mechanism of transformation of the simpler carbon compounds, and the rôle of organic acids in bacterial metabolism can be presented as follows:



<sup>129</sup> Quastel, J. H. *Biochem. Jour.*, 18: 365-380. 1924; 19: 660-666. 1925.

<sup>130</sup> Quastel, J. H. *Biochem. Jour.*, 19: 641-644, 645-651, 652-659, 660-666. 1925. A detailed review of the subject of the transformation of the sugar molecule by bacteria is given by Schoen, M. *Le problème des fermentations*. Masson et Cie. Paris. 1926.

## CHAPTER XVII

### DECOMPOSITION OF PROTEINS AND OTHER ORGANIC NITROGENOUS COMPOUNDS BY SOIL MICROORGANISMS

Most of the nitrogen added to the soil by the plowing under of sod, plant stubble, green and stable manures, is in the form of proteins and their derivatives. The same is true of the organic nitrogenous fertilizers of plant and animal origin, such as dried blood, tankage, fish scraps and cottonseed meal. These substances cannot be assimilated by higher plants as such but have to be first broken down into simple compounds. This process is carried out in the soil by the agency of microorganisms, the final product of hydrolysis being chiefly ammonia. The latter is either used by the plants as such or is oxidized further to nitrates. Nitrates are either assimilated by plants or by microorganisms, reduced by denitrifying bacteria, or washed out in the drainage waters.

The nitrogen content of cereal straw, corn cobs and leaves of trees varies from 0.20 to 0.80 per cent; of legume hay from 1.5 to 3 per cent; of cow manure, free from straw, about 3.5 per cent; horse manure, about 1.5 per cent; chicken manure, 2.1 per cent, on an air dry basis.<sup>1</sup> When these substances are added to the soil they undergo a series of transformations, largely biological in nature, involving processes of hydrolysis, oxidation, reduction and synthesis. These transformations result in the liberation of nitrogen in an available form which may again be wholly or partly reassimilated by soil microorganisms, in the presence of available energy material.

*Physical and chemical properties of proteins.* Proteins are complex substances, consisting of carbon, hydrogen, oxygen, nitrogen, sulfur, and in some cases of phosphorus and iron. The average composition of a typical protein is as follows:

	<i>per cent</i>		<i>per cent</i>
C.....	50.6-55.0	N.....	15.0-19.3
H.....	6.5- 7.3	S.....	0.3- 2.2
O.....	21.5-23.5	P.....	0- 0.9

<sup>1</sup> Thorne, C. E. Farm manures. O. Judd Co., New York. 1914.

No molecular formula can be ascribed to the proteins and their molecular weight ranges from 30,000 to 200,000. Structurally, the proteins are characterized as condensation products of amino acids which are united chiefly by the peptide linkage ( $R-CO \cdot NH-R$ ), similar to the polypeptides. On hydrolysis by acids, alkalis, or specific enzymes, the proteins break up into the constituent amino acids. From 10 to 25 per cent of the nitrogen is present in the proteins in the unstable form of the amide linkage ( $R-CONH_2$ ). About 60 per cent of the nitrogen is assumed to be in the peptide linkage.

The physical properties of the proteins vary very widely. When dried in the presence of moisture, when boiled with acids and alkalis, and when acted upon by some microorganisms, the proteins show a tendency to coloration. This is due to the formation of insoluble pigmented substances, called melanins, probably related to the so-called "humins." Most proteins are soluble in water or in dilute acids or alkalis; a few, like keratin from horn, are insoluble in water and require strong acids and alkalis to bring them into solution. The proteins are amphoteric substances, being capable of combining with both acids and alkalis, neutralizing them, and causing a decrease in the hydrogen- or hydroxyl-ion concentration. Coagulation, precipitation and color reactions vary with the different proteins, depending on their constitution and state of purity.

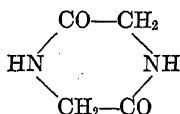
On hydrolysis by acids or enzymes, the proteins are broken down to proteoses, then to peptones and finally to amino acids, which are simple crystallizable substances. Some amino acids, like tyrosine, may appear in the early stages of hydrolysis. The proteoses and peptones consist of several groups of amino acids, these groups being smaller than the original protein. The majority of amino acids in the protein molecule are characterized by the fact that one hydrogen in the  $\alpha$  position is replaced by  $NH_2$ . In the case of two of the basic amino acids, viz., arginine and lysine, a second amino group is present. A third amino acid, histidine, contains an imidazol nucleus and is basic. The general formula of a mono-amino-monocarboxylic acid is  $R-CH(NH_2)COOH$ . When two hydrogens are replaced by amino groups, we have di-amino acids.

Due to the presence of amino groups, the amino acids possess both acid and basic properties, so that glycocoll can form salts both with bases ( $CH_2NH_2COOK$ ) and acids ( $CH_2NH_2COOH \cdot HCl$ ) under proper conditions of hydrogen-ion concentration. The dicarboxylic acids, the general formula of which is  $H_2N \cdot R \cdot (COOH)_2$ , like aspartic and

glutamic acids, possess properties of stronger acids than the mono-carboxylic acids. One of the carboxyls in the dibasic acids is relatively strong, the other is of about the same strength as the carboxyl groups of the ordinary mono amino acids. The three basic amino acids, arginine, histidine and lysine, are fairly strong bases and only show acid properties at extremely small hydrogen-ion concentrations, i.e., pH 12.0 to 14.0. When the hydroxyl group of a carboxylic acid is replaced by an amino group, an acid-amide, like  $\text{CH}_3\text{CONH}_2$  (acetamide) is formed.

Fischer succeeded in combining amino acids into complex groups known as peptides, thus obtaining dipeptides ( $\text{CH}_2\text{NH}_2\text{CO}\cdot\text{NHCH}_2\text{COOH}$  or glycyl-glycine) and other polypeptides, the more complex ones approaching native proteins, in their general properties. The investigations of Fischer and associates gave strong evidence supporting the view that the protein molecule is built up of amino acids in all possible combinations, in the form of a straight chain:  $\text{NH}_2\cdot\text{CHR}\cdot\text{CO}-(\text{NH}\cdot\text{CHR}\cdot\text{CO})_x-\text{NH}\cdot\text{CHR}\cdot\text{COOH}$ . The albumins, globulins, glutelins and gliadins (or prolamins) are the most important vegetable proteins.<sup>2</sup> The chemical nature of the different proteins is determined by the quantitative relationship of the various amino acids and their arrangement in the molecule.

It was later observed, however, that on hydrolysis of proteins by enzymes cyclic compounds were often obtained and not straight polypeptide chains. Abderhalden advanced the theory that dioxopiparazines form, in addition to polypeptides, the elementary units of proteins. Such a ring of two simple amino acids is illustrated by the glycine anhydride:



The pyrrole group as the fundamental unit in the protein structure has also been suggested.<sup>3</sup>

*Chemistry of protein hydrolysis.* Protein decomposition by micro-organisms includes a group of processes; namely, (1) hydrolysis of pro-

<sup>2</sup> Fischer, E. Untersuchungen über Aminosäuren, Polypeptide und Proteine. Berlin. 1906; see Mitchell, N. H. and Hamilton, T. S. The biochemistry of amino acids. Chemical Catalog Co. New York. 1929; Osborne, T. B. The vegetable proteins. Longmans Green & Co. 2nd ed. 1924.

<sup>3</sup> See Klarmann, E. Chem. Rev., 4: 51-107. 1927.

teins to albumoses, peptones and amino acids, (2) deaminization resulting in the formation of ammonia, (3) formation of secondary decomposition products, such as amines, (4) completion of decomposition of proteins involving phenomena of oxidation and reduction with the formation of  $\text{CO}_2$ ,  $\text{H}_2\text{O}$ ,  $\text{H}_2\text{S}$  and  $\text{NH}_3$ .

When the proteins are hydrolyzed by acids and alkalis, the resulting products are amino acids and some ammonia. The latter is probably liberated as a result of the action of the acid or alkali on the amide union in the protein molecule, and in the case of the alkali treatment as a result of the destruction of arginine. The greater portion of the nitrogen is present in the protein molecule as imino groups ( $\text{NH}$ ), with the exception of one of the two amino groups of lysine ( $\omega$  group) which exist free. This is also true of a part of the nitrogen in histidine, arginine and tryptophane. This  $\omega$  group of lysine accounts for the entire amount of amino nitrogen found in the native protein molecule on treating with nitrous acid.<sup>4</sup> The  $\alpha$ -amino groups, which constitute the larger portion of the protein nitrogen, are present only condensed into peptide linkages. On hydrolysis, the free amino nitrogen increases and the peptide linkages ( $\text{R}-\text{CO}\cdot\text{NH}-\text{R}$ ) become separated into amino and carboxyl groups. The measure of this increase in amino nitrogen can serve as an index of the process of protein hydrolysis.

Some proteins are easily hydrolyzed, while others are acted upon with great difficulty. This is very important from the point of view of the availability of nitrogen for plant growth. The action of chemical reagents and moist heat will bring about a complete hydrolysis of the proteins to amino acids and ammonia. Proteolytic enzymes usually do not break down the protein molecule completely. Some, like pepsin, split up the protein chain at one or more junctures, without forming free amino acids, but form groups (albumoses, peptones, peptides) of lower amino acid content. Other enzymes, like trypsin and erepsin, split the protein molecule more completely, bringing about the formation of free amino acids. Still other enzymes (desamidases, deaminases) act upon the amino acids and acid amides liberating ammonia.

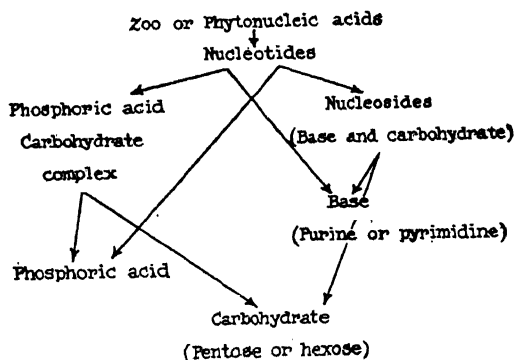
The degradation of proteins by microorganisms proceeds along the general lines followed by acids or proteolytic enzymes. A further transformation of the protein derivatives takes place, however, with the production of various secondary decomposition products, such as ammonia and carbon dioxide, as well as amines, fatty acids, alcohols,

<sup>4</sup> Van Slyke, D. D. and Birchard, F. J. *Jour. Biol. Chem.*, **16**: 539-547. 1913; **10**: 15-55. 1911; **12**: 275-284. 1912; **22**: 281-285. 1915.

aldehydes, methane, phenol, indol, skatol, hydrogen sulfide, etc. Ammonia which is so important, both from the point of view of the metabolism of microorganisms and soil fertility, is usually formed as a secondary decomposition product of the proteins; the amino acid is frequently used thereby as a source of energy.

In some cases proteins form compounds with nucleic acids, giving nucleo-proteins (or protein nucleates). These compounds are present to a limited extent in all plants, animals and microorganisms, and are thus introduced into the soil. On hydrolysis, a nucleo-protein is transformed into an albumin (histone) and nuclein; the nuclein is further hydrolyzed to albumin and nucleic acid. The protein, or albumin, is decomposed by the microorganisms into albumoses, peptones, amino acids and ammonia. The presence of these substances in the soil has actually been demonstrated.<sup>5</sup> Often several groups of organisms take part in the process; some break down the protein to amino compounds and others utilize the latter and form ammonia, as shown later. This is again comparable to the action of the different groups of enzymes.

The composition of nucleic acid from wheat is given as  $C_{41}H_{61}O_{31}N_{16}P_4$ ; that of yeast,  $C_{36}H_{48}O_{30}N_{14}P_4$ . The dissociation products vary with the source of the acid; those of plant origin are phosphoric acid, guanine, adenine, cytosine, thymine and laevulinic acid. Laevulinic acid is formed from a hexose group in the molecule of the nucleic acid. The decomposition of the nucleic acids takes place as follows:<sup>6</sup>



<sup>5</sup> Walters, E. H. Jour. Ind. Eng. Chem., 7: 860. 1915; Lathrop, E. C. Jour. Frankl. Inst., 183: 169-206, 303-321, 465-498. 1917; Shorey, E. C. Jour. Amer. Chem. Soc., 34: 99-107. 1912; Science, 35: 390. 1912; Bur. Soils, U. S. Dept. Agr. Bul. 88. 1912.

<sup>6</sup> Levene, P. A. Abderhald. Handb. Biochem. Arbeitsm., 2: 605-609. 1911; 5: 489-499. 1911.

*Protein decomposition by microorganisms.* The course of protein decomposition by microorganisms can be followed in three different ways.

1. The disappearance of the protein. The residual protein is precipitated by means of an acid or alkali, by alcohol or other precipitating agents, or by heat. The protein is determined either by weighing the dry precipitate or by determining the total nitrogen. In the case of peptone, the biuret test may be used as a measure of its decomposition.<sup>7</sup>

2. The formation of intermediary products, such as peptones or amino compounds. The first can be determined quantitatively by the biuret reaction and the second by the Van Slyke method, the formol titration method, or the Folin method.<sup>8</sup>

3. The formation of ammonia as the final product of protein decomposition.

Each of these three methods has its advantages and disadvantages. By the first method, we determine the absolute amount of protein decomposed, but we do not know how far the decomposition has proceeded, whether to soluble polypeptide molecules or to amino acids and ammonia. The methods of precipitation are also different for the various proteins and involve differences in procedure.

The second method enables one to follow the course of protein decomposition by the increase in the amino nitrogen.<sup>9</sup> The great disadvantage of this method is that the various microorganisms may show different increases of amino nitrogen with the same amount of protein decomposed. This is due to the fact that the various organisms, even decomposing equal amounts of proteins, do not transform the intermediary products with the same rapidity. The fungi, for example, hardly allow any great increase in amino nitrogen, but rapidly transform the intermediary products to ammonia, especially in the absence of available carbohydrate. In the presence of carbohydrate, the protein will be decomposed only to a limited extent. The bacteria and actinomycetes, however, allow a much greater accumulation of amino-nitrogen and a correspondingly lower accumulation of ammonia. The fact that the amino compounds are only intermediate products and that their

<sup>7</sup> Berman, N. and Rettger, L. F. Jour. Bact., 3: 389-402. 1918.

<sup>8</sup> Van Slyke, 1913-14 (p. 414); Sørensen, S. P. L. Biochem. Ztschr., 7: 45. 1907; Folin, O. Jour. Biol. Chem., 51: 377-391. 1922. ~

<sup>9</sup> Sears, H. J. Jour. Inf. Dis., 19: 105-137. 1916; Itano, A. Mass. Agr. Exp. Sta. Bul. 167. 1916; Waksman, 1918 (p. 438); Debord, J. J. Jour. Bact., 8: 7-45. 1923.

accumulation depends on the presence of carbohydrates indicates that, at best, this index can be only approximate.

The third method has the advantage of measuring a final product and not an intermediate one. The fact that the mechanism of ammonia formation from proteins varies with the organisms, some breaking down the protein completely and others incompletely, is an outstanding disadvantage of this method. In the presence of available carbohydrates, more ammonia may also be reassimilated by the organism as a source of nitrogen for the synthesis of its protoplasm, so that a mistaken impression may be had that no protein is decomposed.

The study of protein decomposition would be incomplete without mentioning the so-called processes of "putrefaction," or decomposition of proteins in the absence of oxygen or in the presence of a limited amount of it, with the production of evil smelling gaseous products. This subject has been least studied from the point of view of transformation in the soil; most of the work was done in connection with pathogenic anaerobic bacteria. "Putrefaction" is often differentiated from "decay;" the latter is used to designate the decomposition of nitrogenous organic substances in the presence of oxygen, marked by the volatilization of organic complexes, while the non-volatile mineral constituents are left behind.<sup>10</sup>

Both of these phenomena, namely "putrefaction" and "decay," were not sufficiently understood by the older chemists and bacteriologists. With the advance of our knowledge of the chemistry of proteins, particularly when it was found that ammonia and the "ill-smelling gaseous products" were by-products of secondary reactions following protein hydrolysis, the difference between "decay" and "putrefaction," as indicating activities of special groups of bacteria, disappeared. Like all chemical reactions brought about by biological agencies, the final products of protein decomposition are a result not only of specific microorganisms, but of various environmental conditions, such as oxygen supply and presence of non-nitrogenous substances, which determine the secondary reactions involved after the hydrolysis of the proteins has taken place. The designation of a process by the nature of these secondary reactions, as a result of environmental conditions, led to considerable confusion and to a lack of proper understanding of the processes involved.

<sup>10</sup> Wollny, E. *Die Zersetzung der organischen Stoffe*. Winter, Heidelberg. 1897.



Nencki,<sup>11</sup> who made the first systematic study of the chemical reactions accompanying the decomposition of proteins by bacteria, in the so-called process of putrefaction, found that, in the decomposition of fibrin, albumin and gelatin by bacteria, various products are formed, including leucine, tyrosine, glycocoll and indol. When gelatin was decomposed for four days at 40°C., there were produced, for every 100 parts of gelatin, 9.5 parts of ammonia, 24.2 volatile fatty acids, 12.2 glycocoll, 19.4 peptone and 6.5 carbon dioxide, 71.8 per cent in all. Quantities of gas were also liberated in the process. Nencki concluded that the decomposition of proteins takes place in two stages; viz., processes of hydrolysis, followed by those of reduction and oxidation. Jeannert,<sup>12</sup> studying the decomposition of gelatin under anaerobic conditions, demonstrated, among the products, CO<sub>2</sub>, NH<sub>3</sub>, H<sub>2</sub>S, acetic-, butyric-, and valeric acids, glycocoll and leucine. These substances, with the exception of glycocoll, were also formed from albumin, in addition to hydrogen, hydrogen sulfide, tyrosine and amido-valeric acid.

These investigations were followed by numerous others with crude and pure cultures of bacteria, whereby amino acids, fatty acids and certain gases, including NH<sub>3</sub>, CO<sub>2</sub> and H<sub>2</sub>S, were demonstrated as products of hydrolysis. In the case of the so-called putrefactive processes, indol, skatol and phenol were also demonstrated. *Bac. subtilis* was found<sup>13</sup> to produce, from cotton-seed meal in five weeks, albumoses, peptones, phenyl-acetic and phenyl-propionic acids, ammonia, mercaptan, basic amines, H<sub>2</sub>S, and CO<sub>2</sub>; after three months, valerianic and indol-acetic acids, indol, skatol, and phenol were also demonstrated in the culture. The presence of specific amino acids in the protein molecule is necessary for the formation of some of the final products. It is sufficient to mention tryptophane as a source of indol, cysteine and other sulfur compounds as a source of H<sub>2</sub>S, and tyrosine as a source of cresol and phenol.

A number of bacteria are unable to attack pure proteins; only the degradation products are acted upon. The presence of simple nitrogen compounds may be required to start the development of the organism.<sup>14</sup>

<sup>11</sup> Nencki, M. Ber. deut. Chem. Gesell., 7: 1593-1600. 1874; 8: 336-338. 1875.

<sup>12</sup> Jeannert, J. Jour. prakt. Chem. N. F., 15: 353-389. 1877.

<sup>13</sup> König, J., Spieckermann, A. and Olig, A. Centrbl. Bakt. II, 10: 535-549. 1903.

<sup>14</sup> Bainbridge, F. A. Jour. Hyg., 11: 341-355. 1911; Sperry, J. A. and Rettger, S. F. Jour. Biol. Chem., 20: 445-459. 1915; Berman, N. and Rettger, L. F. Jour. Bact., 3: 367-388. 1918.

In many instances, however, even native proteins will be acted upon after the organism starts to grow and the proper enzymes are formed. Under natural conditions, the proteins are always accompanied by small amounts of their derivatives or by simple nitrogen compounds, especially when added to the soil.

The nature of the products formed depends not only upon the environmental conditions but also upon the organisms concerned. In the great majority of cases, especially in the study of the activities of soil microorganisms, the measurement of ammonia was used as an index of protein decomposition.<sup>15</sup>

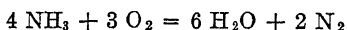
But even in the case of protein decomposition in soil, where one group of organisms readily acts upon the products formed by another, various protein derivatives are found, in addition to ammonia. Lathrop,<sup>16</sup> for example, found that histidine, hypoxanthine, cytosine, xanthine, nucleic acid, creatinine, cyanuric acid are of common occurrence in the soil; arginine, lysine, adenine, choline, trimethylamine occur only infrequently. This led to the assumption that a part of the proteins and other organic nitrogenous compounds are accumulating in the soil, originating from plant residues, stable manure, green manure, organic fertilizers and the bodies of microorganisms. Lathrop analyzed, by the Van Slyke method, soils to which proteins, in the form of dried blood, had been added, at the beginning of the experiment and at the end of various periods of incubation. Even after a 240-day period of decomposition of the dried blood in soil, proteins, or protein-like complexes, insoluble in distilled water, but extractable by dilute alkaline solution, were found to be present in the soil. It is not known, however, whether these proteins are residues from the dried blood which have resisted decomposition by soil microorganisms, or whether they are synthesized materials or constituents of the bodies of the soil organisms. Evidence was obtained to indicate that a formation of new protein material takes place in the soil in the course of decomposition of the proteins and this new protein is perhaps somewhat resistant to further decomposition.

<sup>15</sup> A detailed review of the extensive literature on ammonia formation in the decomposition of organic matter, up to 1910, is given by Voorhees and Lipman, 1907 (p. 433) and Lohnis, 1910 (p. XIV).

<sup>16</sup> Lathrop, 1917 (p. 415); Shorey, E. C. *Jour. Amer. Chem. Soc.*, **34**: 99-107. 1912; **33**: 2035-2042. 1911; *Bot. Gaz.*, **54**: 152-163. 1912; *Bul.* 83, U. S. Dept. Agr.; Morrow, C. A. Thesis, Univ. Minnesota. 1918.

Miyake<sup>17</sup> found that fatty amino compounds seem to be transformed into ammonia more easily than aromatic compounds; aromatic imino compounds are decomposed with greater difficulty than the aromatic amino compounds. The nature of the other group in the molecule does not seem to have any influence upon the rate of transformation of imino nitrogen into ammonia nitrogen.

These results tend to indicate that a simple observation of the amount and rate of ammonia formation need not necessarily indicate the course of protein decomposition. A certain set of conditions will lead to the formation of one group of compounds by bacteria from a certain protein, while another group of compounds will be formed from the same protein under different conditions. It was also generally assumed<sup>18</sup> that the rapid oxidation of proteins may result in the liberation of elementary nitrogen, according to the reaction:



However, the work of Ehrenberg<sup>19</sup> pointed to the negligible loss of nitrogen from this source. Appreciable losses may occur either through direct volatilization of ammonia or the leaching of nitrates.

*Chemistry of ammonia formation in the decomposition of proteins by microorganisms.* Müntz<sup>20</sup> was the first to demonstrate in 1890 that organic matter is decomposed by organisms with the formation of ammonia, which is only then nitrified. In soils in which nitrification has been stopped by the use of heat or disinfectants, ammonia accumulates, indicating that this treatment was sufficient to kill the organisms oxidizing ammonia to nitrate, but not those that produce ammonia from proteins. Müntz and Coudon have further shown that no ammonia was formed during two and one-half years in sterilized soil, while the unsterilized soil produced, in sixty-seven days, 41 to 100 mgm. of ammonia per 100 grams of soil. These investigations were followed by those of Marchal<sup>21</sup> and numerous others which pointed to the importance of ammonia formation in the soil and the rôle of microorganisms in its formation from proteins. This was found to be not

<sup>17</sup> Miyake, K. Jour. Amer. Chem. Soc., **39**: 2378-2382. 1917.

<sup>18</sup> Voorhees and Lipman, 1907 (p. 433), p. 49.

<sup>19</sup> Ehrenberg, 1907 (p. 251).

<sup>20</sup> Müntz, A. Compt. Rend. Acad. Sci., **110**: 1206-1209. 1890; Müntz, A. and Coudon, H. Compt. Rend. Acad. Sci., **116**: 395-398. 1893.

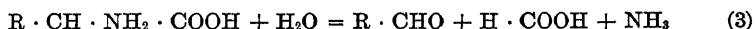
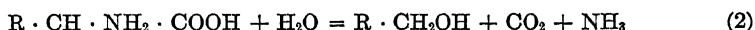
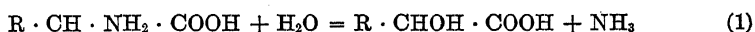
<sup>21</sup> Marchal, E. Bull. Acad. Roy. Sci. Belg., (3), **25**: 728-738; **27**: 71-103. 1895; Centrbl. Bakt. II, **1**: 753-758. 1895.

a specific property of certain bacteria, but a function of a large number of microorganisms.

When proteins are hydrolyzed by means of acids or enzymes, only 10 per cent of the total nitrogen in casein and 25 per cent in gliadin is liberated as ammonia, as a result of the breaking of the acid amide ( $-\text{CO}\cdot\text{NH}_2$ ) linkages. When proteins are acted upon by microorganisms, especially when they are used as sources of energy, large quantities of ammonia are produced as a waste product. Seventy-five per cent or more of the protein-nitrogen can be found to accumulate in the soil in the form of ammonia within a few days, with proteins as the only source of energy. The ammonia is produced by a series of chemical changes which depend upon the nature of the organism, presence of nutrients other than amino acids, such as available carbohydrates, upon oxygen tension and other conditions under which the reactions take place.

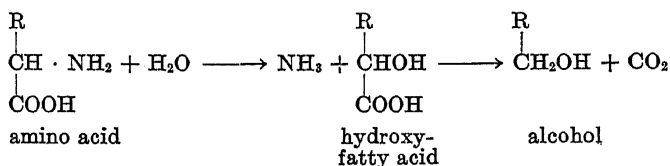
Ammonia formation from amino acids may involve processes of hydrolysis, oxidation or reduction, or a combination of two or all, resulting in the splitting of the amino or carboxyl groups or both. The various reactions may be summarized, as follows:

✓1. Hydrolytic deamination. The hydrolysis of an amino acid may result in the formation of a lower fatty acid and ammonia, or of an alcohol,  $\text{CO}_2$  and ammonia, or of an aldehyde, lower acid and ammonia, as shown by the general formulae:

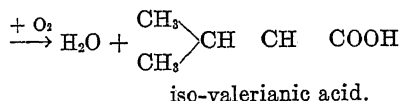
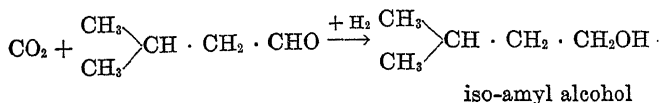
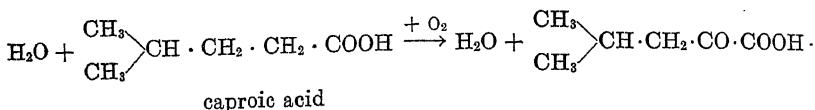
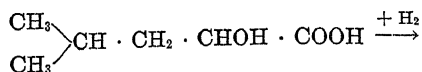
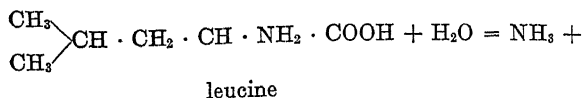
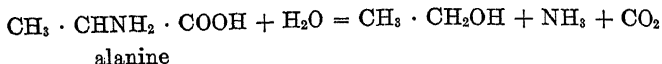


These processes are carried out by various aerobic organisms. Formula (2) is of common occurrence among bacteria, fungi and yeasts, as in the case of formation of isoamyl alcohol from leucine.<sup>22</sup>

According to Ehrlich, the reaction takes place as follows:



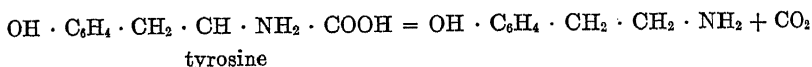
<sup>22</sup> Dakin, H. D. Jour. Biol. Chem., 4: 63-76. 1908; Ehrlich, F. and Jacobsen, K. A. Ber. deut. Chem. Gesell., 44: 888. 1911; Nawiasky, P. Arch. Hyg., 66: 209-243. 1908; also 64: 33-61. 1908; Ehrlich, F. Ztschr. Ver. Deut. Zuckerind. Tech. T. N. S., 42: 539-567. 1905.



## 2. Decarboxylation with amine formation:



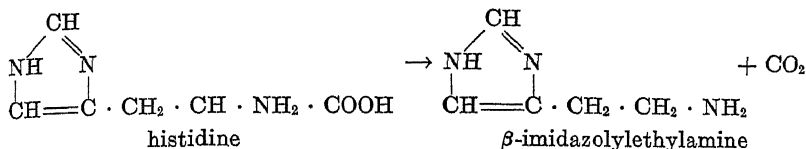
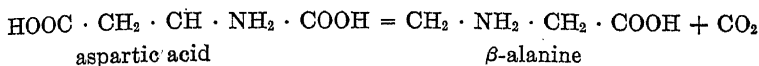
This process of amino acid decomposition through the amine stage, with the formation of alcohol and ammonia, has been described for yeasts and fungi.<sup>23</sup> The first part of the process, namely the formation of amines, is characteristic of the so-called putrefaction processes.<sup>24</sup> The transformation of amino acids into nitrogen bases is found to take place in the formation of para-oxy-phenyl-ethylamine from tyrosine, of pentamethyl-diamine (cadaverin) from lysine, of tetramethylenediamine (putrescin) from ornithine, etc.



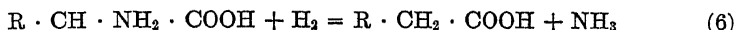
<sup>23</sup> Ehrlich, F. Ber. deut. chem. Gesell., **39**: 4072. 1906; **40**: 1027. 1907; **44**: 139. 1911; **45**: 883. 1912; Biochem. Ztschr., **2**: 52. 1906; Ehrlich, F. and Jacobsen, K. A. Ber. deut. chem. Gesell., **44**: 888. 1911; Ackermann, O. Ztschr. Biol., **56**: 87. 1911; Ehrlich, F. and Pistchimuka, P. Ber. deut. chem. Gesell., **45**: 1006-1012. 1912.

<sup>24</sup> Guggenheim, M. Biogene Amine. Urban & Schwarzenberg. 1923; Abderhalden's Handbuch biol. Arb. Meth. Abt. I, **7**: 295-582. 1928. Rettger, L. F. Jour. Biol. Chem., **2**: 71-96. 1906; **4**: 45-55. 1907; **13**: 341-346. 1912.

A mono-amino di-carboxylic acid may lose a CO<sub>2</sub> group, with the formation of a mono-carboxylic acid:



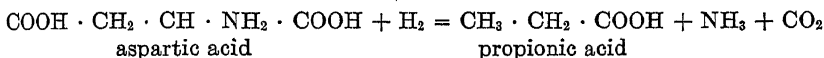
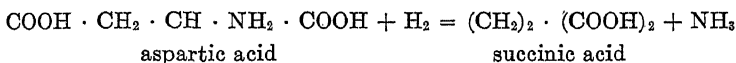
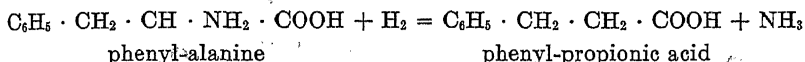
### 3. Reductive deaminization:



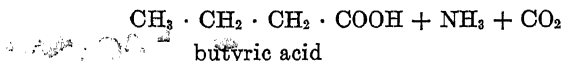
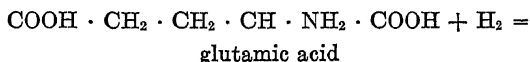
or



This process of reduction is carried on by anaerobic bacteria, which reduce the α-amino acids, with the formation of saturated fatty acids and ammonia. As instances, one may cite the formation of acetic acid and ammonia, or methane, CO<sub>2</sub> and ammonia, from glycocoll, as well as the following reactions:



The formation of butyric acid takes place according to the same reaction:

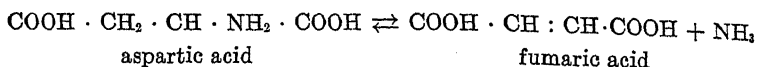


4. Anaerobic bacteria may produce ammonia from amino acids, without reduction.<sup>25</sup>

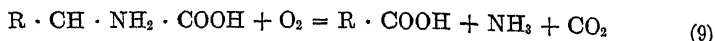


<sup>25</sup> Raistrick, H. Biochem. Jour., **13**: 446-458. 1919; Cook, R. C. and Woolf, B. Ibid., **22**: 474-481. 1928.

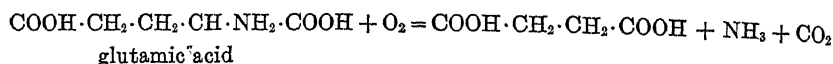
The reaction may be reversible, i.e. the bacteria may bring about both deamination and synthesis:



#### 5. Oxidative deamination:

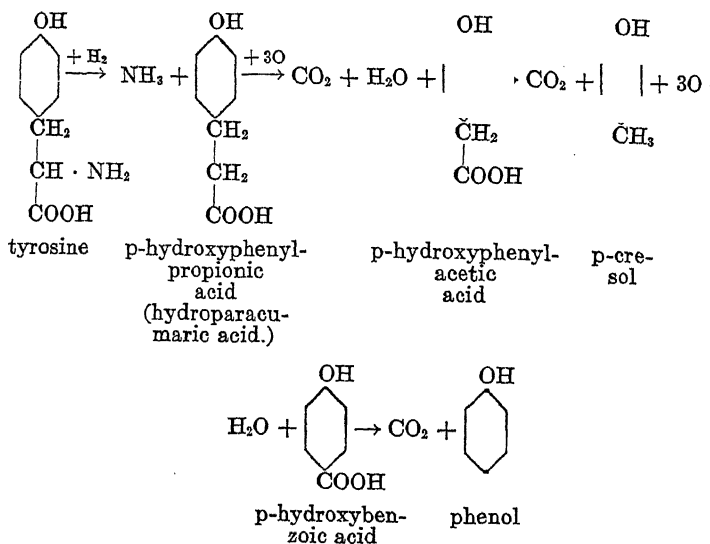


This process is carried out by aerobic organisms, especially by fungi.<sup>26</sup> As examples of this reaction, the transformation of leucine into isovaleric acid,<sup>27</sup> as shown above, as well as of glutamic acid into succinic acid may be cited:



The decomposition of one amino acid may involve the reactions of oxidation, deamination, decarboxylation and reduction. The same organism may bring about a series of these reactions, while different results may be obtained by the same organism under different conditions.

The transformation of tyrosine takes place according to the following reactions:



<sup>26</sup> Dakin, H. D. Oxidations and reductions in the animal body. 2nd ed., Longmans, Green & Co. 1922; Ehrlich and Jacobsen, 1912 (p. 422); Ehrlich, F. Biochem. Ztschr., 2: 52-80. 1906; Neubauer, O. and Fröschers, K. Ztschr. Physiol. Chem., 70: 326-350. 1911; Janke, A. Arch. Mikrob., 1: 304-332. 1930.

<sup>27</sup> Nencki, M. Jour. prakt. Chem., 17: 105-124. 1878.

Tyrosine may also be decomposed to homogentisic acid, ammonia and carbon dioxide, as shown by Beijerinck<sup>28</sup> for an actinomyces. Bacteria belonging largely to the colon-typhoid group have been shown to produce histamine from histidine and tyramine from tyrosine.<sup>29</sup> In the decomposition of tryptophane, the bacteria are also capable of breaking down the indol ring and use the nitrogen of the nucleus.<sup>30</sup>

Aeration conditions have an important influence upon the nature of the products formed from the decomposition of the amino acids. Hydroxy acids, formed under aerobic conditions, may prove unstable under anaerobic conditions; p-hydroxy-phenyl-propionic acid formed from tyrosine under anaerobic conditions is oxidized to p-cresol and phenol when air is admitted.

The acids formed in the process of deamination give rise to calcium salts. These are broken down to carbonates and the ammonia is oxidized to nitrates. The amines formed through the process of decarboxylation are, however, more resistant to bacterial action. Glucose-amine is readily used both as a source of carbon and as a source of nitrogen. Pentamethylenediamine and ethanolamine are utilized to a more limited extent. Most of the other amines can be used by bacteria as sources of nitrogen in the presence of available carbohydrates. Lower amines (ethyl- and methyl-amine) or protamines can be decomposed only by bacteria isolated by the elective culture method; these bacteria are referred to as protaminophagous organisms (*Protaminobacter*).<sup>31</sup>

Of the optically active amino acids, both forms are attacked, in varying proportions, by bacteria; in the case of glutamic acid, the rate of decomposition is the same.<sup>32</sup> *Bact. pyocyaneum* attacks readily aliphatic and cyclic amino acids as sources of energy, but not benzene derivatives (p- or m-amino-benzene). It decomposes tyrosine completely; tryptophane is broken down to  $(\text{NH}_4)_2\text{CO}_3$  and indol; the indol is converted to anthranilic acid. In the decomposition of amino acids by *Bact. pyocyaneum*, the carboxyl group is first removed and

<sup>28</sup> Beijerinck, M. W. K. Akad. Wetenschappen Amsterdam., 15: 932-937. 1913.

<sup>29</sup> Koessler, K. K., Hanke, M. T. and Sheppard, M. S. Jour. Inf. Dis., 43: 363-377. 1928.

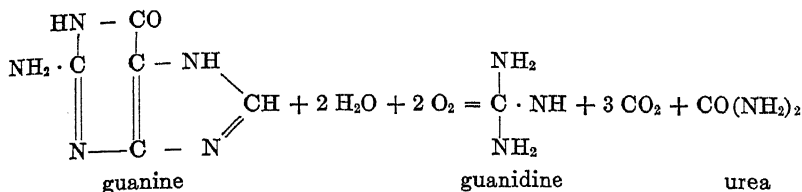
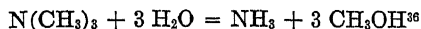
<sup>30</sup> Raistrick, H. and Clark, A. B. Biochem. Jour., 15: 76. 1921.

<sup>31</sup> de Jong, L. E. D. Centrbl. Bakt. II, 71: 193-232. 1927; Guggenheim. Die biogene amine. 1924; Hirsch, P. Die Einwirkung von Mikroorganismen auf die Eiweisskörper. Berlin. 1918.

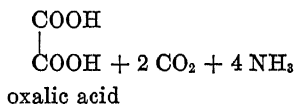
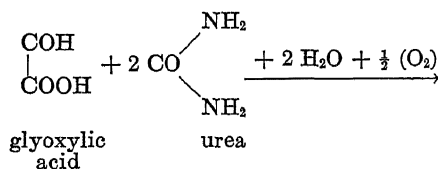
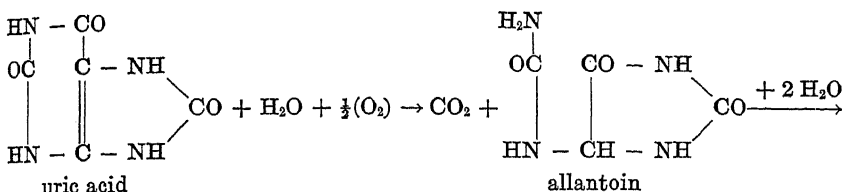
<sup>32</sup> Neuberg, C. Biochem. Ztschr., 18: 431-434. 1909.



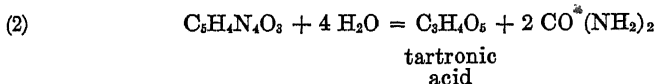
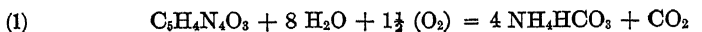




Uric acid undergoes a series of transformations before ammonia is produced, both in animal metabolism, and in its decomposition by bacteria.<sup>37</sup>



Uric acid can also be decomposed by various bacteria according to the following reactions:<sup>38</sup>



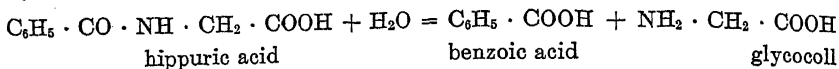
<sup>36</sup> Ehrlich, F. and Lange, F. Ber. deut. chem. Gesell., **46**: 2746. 1913.

<sup>37</sup> Sestini, F. and L. Landw. Vers. Sta., **38**: 157-164. 1890; Wiechowski, W. Biochem. Ztschr., **25**: 431-459. 1910; Liebert, F. K. Akad. Wetensch. Amsterdam. Proc. Sect. Sci., **12**: 54-64. 1909; Nawiaskey, P. Arch. Hyg., **66**: 241. 1908.

<sup>38</sup> Gerard, E. Compt. Rend. Acad. Sci., **122**: 1019-1022; **123**: 185-187. 1896; Ulpiani, C. and Cingolani, M. Gaz. chim. Ital. II, **33**: 93-98, 98-124. 1903 (Chem. Centrbl., **2**: 1287. 1903).

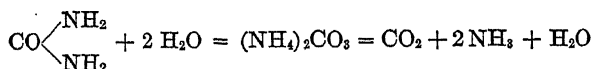
Many bacteria are capable of producing uric acid in the process of protein decomposition.<sup>39</sup>

A large number of microorganisms are able to use hippuric acid as a source of carbon and nitrogen. The acid is usually first hydrolyzed by an enzyme produced by these organisms:<sup>40</sup>



A number of bacteria (*B. megatherium*, *B. subtilis*, *B. mesentericus*) and actinomyces produce urea in the decomposition of proteins. Some bacteria (*B. fluorescens*, *B. coli communis*) are capable of forming urea only in media containing arginine. In the absence of carbohydrates, urea accumulates when the organism is unable to form the enzyme urease. In the presence of carbohydrates, urea is not formed. Many higher fungi contain considerable quantities of urea, which is a by-product in the metabolism of these fungi as it is in the case of higher animals.<sup>41</sup>

The rapidity of the transformation of urea to ammonia depends on the temperature and the abundance of organic matter in the soil. The addition of manure to soil, in which urea is only slowly hydrolyzed, hastens the process. Rapid transformation of urea is necessary in order to avoid a loss of nitrogen, due to the coexistence of urea, nitrous acid and nitric acid at the same time. Only a part of the urea is changed in the decomposition to ammonia, while a part is changed to organic compounds.<sup>42</sup> Urea is hydrolyzed, with the formation of ammonia, by a large number of soil microorganisms as well as by specific groups of bacteria, which utilize the energy liberated in the process:<sup>43</sup>



The decomposition of urea is believed to take place in two definite stages with the formation of ammonium carbonate as the intermediary

<sup>39</sup> McDonald, J. F. Levine, V. E. and Gleason, M. Amer. Jour. Physiol., 78: 437-448. 1926; Schlossmann, K. Centrbl. Bakt. I, 110: 78-84. 1929.

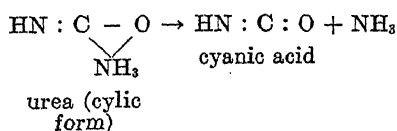
<sup>40</sup> Kossowicz, A. Ztschr. Gärungsphysiol., 1: 60-62. 1912.

<sup>41</sup> Ivanov, N. N. Ztschr. physiol. Chem., 170: 274-288. 1927; Biochem. Ztschr. 181: 8-16. 1927.

<sup>42</sup> Bordas, J. and Mathieu, G. Ann. Sci. Agron., 47: 711-727. 1930.

<sup>43</sup> A detailed study of the chemistry of urea is given by E. A. Warner The Chemistry of Urea. Longmans, Green & Co., New York. 1923; decomposition of urea in soil by T. Gibson. Jour. Agr. Sci., 20: 549-558. 1930; Centrbl. Bakt. II, 81: 45-60. 1930.

product.<sup>44</sup> The enzyme urease decomposes urea first into cyanic acid and ammonia:



Cyanic acid is hydrolyzed, in the presence of water:



However, this process still remains to be confirmed. According to Söhngen,<sup>45</sup> urea offers an exclusive source of energy to the urea bacteria

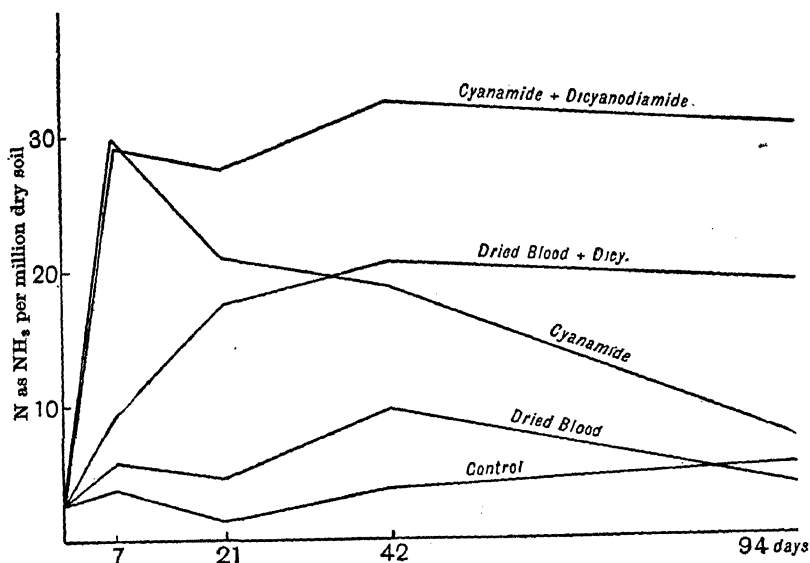


FIG. 20. Accumulation of ammonia from cyanamide and dried blood, as influenced by the presence of dicyanodiamide (from Cowie).

but not a source of carbon, so that a carbohydrate is also necessary to insure a growth of the organisms. The maximum hydrolysis of urea, however, accompanies a minimum oxidation of organic compounds.

<sup>44</sup> Yamazaki, E. Jour. Tokyo Chem. Soc., **39**: 125-184. 1918; Sci. Rept. Tohoku Imp. Univ., **9**: 97, 136. 1920; Fearon, W. R. Biochem. Jour., **17**: 84-93. 1923; Physiol. Rev., **6**: 399-439. 1926.

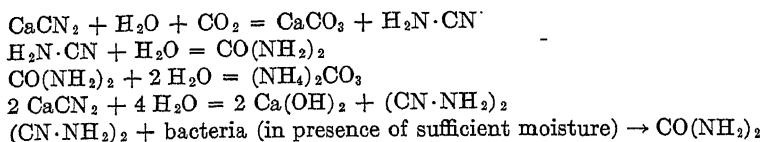
<sup>45</sup> Söhngen, N. L. Centrbl. Bakt. II, **23**: 91-98. 1909

*B. erythrogenes*, for example, hydrolyzes 500 mgm. urea for every 20 mgm. of carbon assimilated, while *Urobac. jakschii* hydrolyzes 1800 mgm. of urea for 10 mgm. of carbon assimilated.

Cyanamide readily breaks down in the soil yielding ammonia which is then nitrified practically quantitatively.<sup>46</sup> Cyanamide may first be decomposed in the soil into urea by a purely chemical process,<sup>47</sup> under the influence of catalyzers, or it may polymerize into dicyanodiamide (especially in the presence of catalyzers such as  $\text{ZnCl}_2$ ).

Dicyanodiamide is not toxic to fungi, ammonia forming bacteria, animals and plants grown in water cultures, but is toxic to plants grown in soil (non-toxic effect in sterile soil). This effect is ascribed to the formation by the bacteria of a toxic substance, namely guanidin and  $\text{HCN}$ . However, the last compound is decomposed by bacteria in soil.<sup>48</sup> Certain bacteria can actually use dicyanodiamide as a source of nitrogen, in the presence of glucose; this amide is decomposed, however, only to a very inappreciable extent and no ammonia is formed.<sup>49</sup>

When cyanamide is added to sterile soil no ammonia is formed, but considerable amounts of ammonia are produced on the addition of urease; this indicates the formation of urea. The urea is, of course, decomposed in the soil by various organisms. The process of decomposition of cyanamide can be thus presented as follows:<sup>50</sup>



Indol ( $\text{C}_6\text{H}_4\text{CH} : \text{CH}\cdot\text{NH}$ ) is widely distributed in nature; it is present in plants and it is also produced in the anaerobic decomposition of proteins by bacteria. Various bacteria are capable of decomposing indol.

<sup>46</sup> Löhnis, F. and Sabashnikoff, A. Centrbl. Bakt. II, 20: 322-332. 1908; Vermeire, M. Naturwetensch. Tijds., 9: 40-44. 1927; Walton, J. H. Mem. Dept. Agr. India, Bact. Ser., 3: 35-64. 1928.

<sup>47</sup> Cowie, G. A. Jour. Agr. Sci., 9: 113-136. 1919; 10: 163-176. 1920; Garby, C. D. Jour. Ind. Engin. Chem., 17: 266-268. 1925.

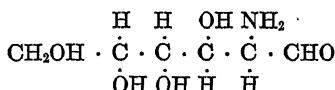
<sup>48</sup> Norris, R. V. et al. Mem. Dept. Agr. India, Pusa., 7: 55-75. 1923; Ulpiani, C. Gaz. chim. Ital., 40: 613-666. 1910; Loew, O. Centrbl. Bakt. II, 70: 39-41. 1927; Aslander, A. Bot. Gaz., 85: 462-463. 1928.

<sup>49</sup> Perotti, R. Centrbl. Bakt. II, 21: 200-231. 1908; Centrbl. Bakt. II, 20: 514-518. 1908; 24: 373-382. 1909; Löhnis, 1910 (p. XIV), p. 590.

<sup>50</sup> Einosuke, T. Jour. Soc. Chem. Ind. Japan, 30: 343. 1927.

*Bact. pyocyaneum* forms out of indol anthranilic (*o*-aminobenzoic) acid.<sup>51</sup> According to Gray,<sup>52</sup> certain soil bacteria are capable of oxidizing indol to indigotin. The latter is formed, within 20 hours, on an agar medium, outside of the organism, in the form of crystals. A source of carbon is essential, since indol cannot be used as such; no indoxyl was found in the cultures, while the indigotin cannot be oxidized to isatin.

Chitin is found among the synthesized constituents of the cells of microorganisms, especially fungi, and is constantly added to the store of soil organic matter. It consists of one molecule of glucoseamine and three molecules of acetyl-glucosamine, from which four molecules of water have been removed ( $C_{30}H_{50}O_{19}N_4$ ). Another formula for chitin has been suggested:



Chitin gives a violet color with chlor-zinc iodide and a brown-red color with a solution of iodine and potassium iodide. It is insoluble in water, in ammoniacal copper solution and in 10 per cent alkali solution, but is hydrolyzed with concentrated hydrochloric acid to give glucoseamine. Certain bacteria and actinomycetes decompose chitin in the soil by means of an enzyme *chitinase*.<sup>53</sup> Chitin can be used by these organisms both as a source of carbon and nitrogen, in the presence of  $K_2HPO_4$  and  $MgSO_4$ .

A number of other nitrogenous compounds, such as the various alkaloids, are subject to decomposition by microorganisms.<sup>54</sup> Ammonia or nitrate formation can be used as an index of decomposition of these various compounds. It was found<sup>55</sup> that diamino-mono-carboxylic acids are changed into nitrates more readily than the mono-amino acids; the mono-amino-dicarboxylic acids are decomposed somewhat more readily than the mono-carboxylic acids. Hippuric acid approaches in the rapidity of its transformation into nitrate to the mono-amino mono-carboxylic acids. Xanthine and uric acid were more readily decomposed than all other organic compounds tested, except nicotine and arginine.

<sup>51</sup> Supniewski. Biochem. Ztschr., **146**: 522. 1924.

<sup>52</sup> Gray, P. H. H. Proc. Roy. Soc. B., **102**: 263-280. 1928.

<sup>53</sup> Benecke, W. Bot. Ztg., **63**: 227-272. 1905; Folpmers, T. Chem. Weekbl., **18**: 249. 1921; (Centrbl. Bakt. II, **57**: 97-98. 1922).

<sup>54</sup> Lavalie, P. Bull. Sci. pharmacol., **30**: 321-325. 1923; (Chem. Abstr., **17**: 2732).

<sup>55</sup> Batham, H. N. Soil Sci., **24**: 187-204. 1927.

Brucine and strychnine had a temporary toxic effect upon nitrate formation. A fairly close correlation was found between the degree of nitrification and the carbon nitrogen ratios of the specific compounds.

*Ammonia formation by bacteria.* The earlier investigators of bacterial metabolism, like Hoppe-Seyler, Bienstock, Hauser and others, found that mixtures and pure cultures of bacteria, like *Bact. vulgare*, *Bac. subtilis*, *Bact. prodigiosum*, *Bac. putrificus*, *Bact. fluorescens liquefaciens*, are capable of breaking down proteins with the formation of various end products, one of which was ammonia. Proteins of both plant and animal origin are decomposed by a number of bacteria giving a great variety of products.

Marchal used a solution containing 1.5 per cent nitrogen, in the form of egg albumin made insoluble by means of 0.01 per cent ferric sulfate,

TABLE 36

*Transformation of protein nitrogen into ammonia by microorganisms*

	PER CENT		PER CENT
<i>Bac. mycoides</i> .....	46	<i>Bac. arborescens</i> .....	19
<i>Bact. vulgare</i> .....	36	<i>Bact. fluorescens liquefaciens</i> ....	16
<i>Bac. mesentericus vulgatus</i> .....	36	<i>Cephalothecium roseum</i> .....	37
<i>Sarcina lutea</i> .....	27	<i>Asp. terricola</i> .....	32
<i>Bac. subtilis</i> .....	23	<i>Botryotrichum piluliferum</i> .....	24
<i>Bac. janthinus</i> .....	23	<i>Stemphylium</i> .....	5
<i>Bact. fluorescens putidum</i> .....	22	<i>Actinomyces</i> .....	21

which was inoculated with various bacteria; ammonia was determined after 20 days' incubation at 30° by distilling with MgO (table 36).

The various strains of *Bac. mycoides* derived from different sources varied in their power to produce ammonia from proteins. In the case of one strain, Marchal obtained a transformation of 58 per cent of egg-albumin nitrogen into ammonia, accompanied by a marked change of the reaction of the medium to alkaline. The more dilute the solution of the protein the greater was the transformation. Of the individual amino acids, 66 per cent of the nitrogen in tyrosine, using a 0.4 per cent solution of the acid, some sugar and salts, was transformed into ammonia; 40 per cent of leucine and 37 per cent of asparagine in a 1 per cent solution were changed to ammonia; only 9 per cent of the creatine was transformed into ammonia. In addition to CO<sub>2</sub> and NH<sub>3</sub>, peptone, leucine, tyrosine, some formic, propionic and butyric acids were demon-

strated among the products of the digestion of albumin by bacteria. For every milligram of ammonia formed 8.9 mgm.  $\text{CO}_2$  were liberated. Marchal concluded that *Bac. mycoides* is one of the most common soil organisms and one that attacks proteins most energetically. It is favored by a temperature of  $30^\circ$ , complete aeration, slightly alkaline medium and a slight concentration of nitrogenous substance in solution.

These studies were confirmed and further extended by numerous investigators.<sup>56</sup> The great majority of soil organisms developing on the plate were found to produce ammonia from proteins. The gelatin-liquefying bacteria are capable of inducing a greater protein decomposition with a more abundant ammonia formation. Since this group

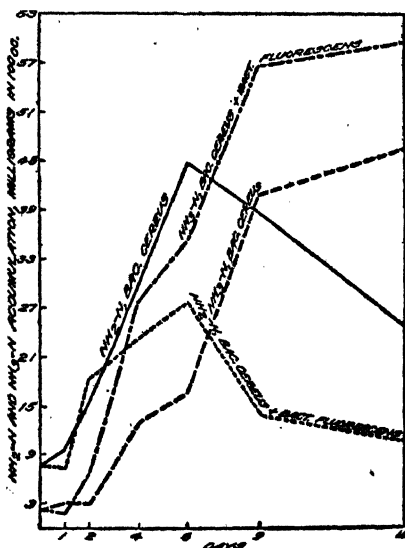


FIG. 21. Course of accumulation of amino- and ammonia-nitrogen from casein by *Bac. cereus* and *Bact. fluorescens* (from Waksman and Lomanitz).

forms at times more than 15 per cent of the total number of soil bacteria developing on the plate, they were believed to do the initial work in rendering soluble the protein nitrogen in the soil, so that it might be

<sup>56</sup> Severin, S. A. Centrbl. Bakt. II, 1: 97-104, 160-168, 799-817. 1895; 628-633. 1897; 7: 369-386. 1901; 13: 616-631. 1904; Chester, F. D. Delaware Sta. Bul. 65. 1904; Löhnis, 1905 (p. 116); Lipman, J. G. N. J. Agr. Exp. Sta. 19th Ann. Rpt. 1906, 119-188; Gage, S. D. Jour. Amer. Chem. Soc., 27: 327-363. 1905; Voorhees, E. B. and Lipman, J. G. Bul. 194, Office Exp. Sta., U. S. Dept. Agr. 1907.



further decomposed by the same or other soil organisms. Lipman and Burgess<sup>57</sup> tested a series of pure cultures of bacteria for their ammonia-producing power, using various nitrogenous substances in various soils. *Bac. tumescens* was found to be the most efficient organism of all tested, although in some cases greater efficiency was obtained for *Bac. mycoides* and *Sarcina lutea*. Usually 20 to 30 per cent of the protein nitrogen was transformed into ammonia in twelve days.

According to Conn,<sup>58</sup> however, the non-spore forming bacteria are much more active in manured soil than the spore forming organisms. *Bac. cereus* was found<sup>59</sup> to decompose proteins to amino acids, while *Bact. fluorescens* acts largely upon amino acids. In the presence of a mixture of the two organisms, the protein is rapidly changed to ammonia. Thus there is a possibility that different organisms take an active part in different stages of the process of protein decomposition; bacteria like *Bac. cereus* may be active in the first stages of hydrolysis and bacteria like *Bact. fluorescens*, in the latter stages leading to the formation of ammonia (fig. 21). This confirmed the earlier investigations of Tissier and Martelly,<sup>60</sup> who found that the action of *Bact. coli*, of various micrococci and of *Bact. filiformis aerobius* upon natural proteins was nil, or almost nil; but they acted very readily upon the hydrolytic products of proteins. The same was true of certain anaerobic bacteria and even the action of *Bact. vulgare* upon pure proteins has been doubted. However, various spore-forming bacteria, especially anaerobes, like *Bac. gracilis putidus*, *Bac. putrificus* and also *Bac. perfringens*, *Bac. bifermentans* and *Bac. sporogenes* rapidly decompose native proteins. The anaerobic bacteria vary considerably in their ability to attack the individual amino acids.<sup>61</sup>

The rapidity of ammonia formation from proteins by bacteria depends not only upon the nature of the organism but also upon the kind of protein. The process of ammonia formation was completed in a few days in the case of casein, while it continued, even after a month, with gliadin.<sup>62</sup> The amino-nitrogen content of the gliadin and casein media was 0.57 and 0.68 mgm. before hydrolysis; 42.56 and 99.31 mgm. after

<sup>57</sup> Lipman, C. B. and Burgess, P. S. Univ. Cal. Publ. Agr. Sci., 1: 141-172. 1914.

<sup>58</sup> Conn and Bright, 1919 (p. 43).

<sup>59</sup> Waksman and Lomanitz, 1925 (p. 365).

<sup>60</sup> Tissier, H. and Martelly. Ann. Inst. Past., 16: 865-903. 1902.

<sup>61</sup> Mead, M. W. and King, C. G. Jour. Bact., 17: 151-161. 1929.

<sup>62</sup> Robinson, R. H. and Tartar, H. V. Jour. Biol. Chem., 30: 135-144. 1917.

acid hydrolysis, and 17.03 and 46.00 after hydrolysis with *Bac. subtilis*. All the nitrogen forms of the protein molecule are changed more or less by the action of bacteria, the end product being ammonia; in no case, however, is one form of nitrogen completely destroyed. A similarity was found in the chemical change produced by acid hydrolysis and bacteria.

*Ammonia formation by fungi and actinomyces.* The actinomyces develop on artificial culture media and in the soil slower than the fungi and, when a short period of incubation is used, their intense activity in breaking down proteins and forming ammonia may be overlooked. When a long period of incubation (30 days or more) is employed, they are found to be very active in this respect.<sup>63</sup> The important point in this connection is that these organisms are capable of allowing a large accumulation of ammonia even in the presence of available carbohydrates; in other words, they prefer proteins to carbohydrates as sources of energy. According to Guittonneau,<sup>64</sup> actinomyces produce from proteins, not only ammonia, but also urea, both in the presence and absence of glucose.

Fungi are able to decompose proteins very vigorously. Different species vary greatly in this respect; the nature of the protein, reaction of medium and presence of available carbohydrates affect the process. A large part of the nitrogen may be left in the form of intermediary products. Organisms like *Asp. niger*, which produce large amounts of acid (oxalic and citric) from carbohydrates and even from proteins and which are thus enabled to neutralize the ammonia, accumulate only very small amounts of amino acids in artificial cultures; at the same time appreciable quantities of ammonia are formed in the medium. But when the oxalic acid is neutralized with  $\text{CaCO}_3$ , or when the formation of both oxalic acid and ammonia is prevented by means of insufficient aeration, an accumulation of amino acids takes place.<sup>65</sup>

When the protein is the only source of carbon available for fungi, a definite parallelism is found between the growth of the mycelium and the production of ammonia. Different protein derivatives are not utilized alike and their nitrogen is not liberated alike in the form of ammonia. *Asp. niger* grows best with leucine, followed by peptone,

<sup>63</sup> Fousek, 1912 (p. 289); Macé, E. *Compt. Rend. Acad. Sci.*, **14**: 147-148. 1905; Waksman, 1920 (p. 286).

<sup>64</sup> Guittonneau, G. *Compt. Rend. Acad. Sci.*, **178**: 1383-5. 1924.

<sup>65</sup> Butkewitsch, W. *Jahrb. wiss. Bot.*, **38**: 147-240. 1903. *Rec. d'articles dédiés au Prof. C. Timiriazeff*. 1916, 457-499.

asparagine and glycocoll. The difference in the nature of the carbon compounds either accompanying the proteins or the protein carbon itself accounts for the difference in the amount of fungus growth and ammonia formation. This is due to the fact that, in the absence of available carbohydrates, the fungus uses the protein both as a source of energy and as a source of nitrogen; the amount of nitrogen liberated

TABLE 37  
*Ammonia formation by soil fungi*

ORGANISM	SOURCE OF NITROGEN*	MILLIGRAMS OF NH <sub>4</sub> N
<i>Pen. intricatum</i> .....	D. B.	20.45-11.26
<i>Pen. intricatum</i> .....	C. S. M.	4.65- 3.85
<i>Pen. chrysogenum</i> .....	D. B.	21.81
<i>Pen. chrysogenum</i> .....	C. S. M.	16.82
<i>Asp. fumigatus</i> .....	D. B.	12.16
<i>Asp. fumigatus</i> .....	C. S. M.	7.91
<i>Mucor hiemalis</i> .....	D. B.	12.75-29.94
<i>Mucor hiemalis</i> .....	C. S. M.	18.09-25.89
<i>Rhiz. nigricans</i> .....	D. B.	12.65
<i>Rhiz. nigricans</i> .....	C. S. M.	25.40
<i>Zyg. vuilleminii</i> .....	D. B.	28.73
<i>Zyg. vuilleminii</i> .....	C. S. M.	31.43
<i>Monilia sitophila</i> .....	D. B.	19.51
<i>Monilia sitophila</i> .....	C. S. M.	40.23
<i>Trich. koningi</i> .....	D. B.	76.48-66.16
<i>Trich. koningi</i> .....	C. S. M.	42.60-30.63

\* To 100 grams of soil were added 155 mgm. of nitrogen in the form of D. B. = dried blood or of C. S. M. = cottonseed meal.

as ammonia depends not only upon the nitrogen content of the protein, but largely upon the availability of the carbon; the nitrogen is then either liberated as a waste product, ammonia, or is reassimilated and changed into microbial protein.

McLean and Wilson<sup>66</sup> concluded that fungi, rather than bacteria,

<sup>66</sup> McLean, H. C. and Wilson, G. W. N. J. Agr. Exp. Sta., Bul. 270. 1914.

are responsible for the large accumulations of ammonia in soil rich in organic nitrogenous substances and that this depends upon the chemical and physical composition of the soil, quality of the organic matter

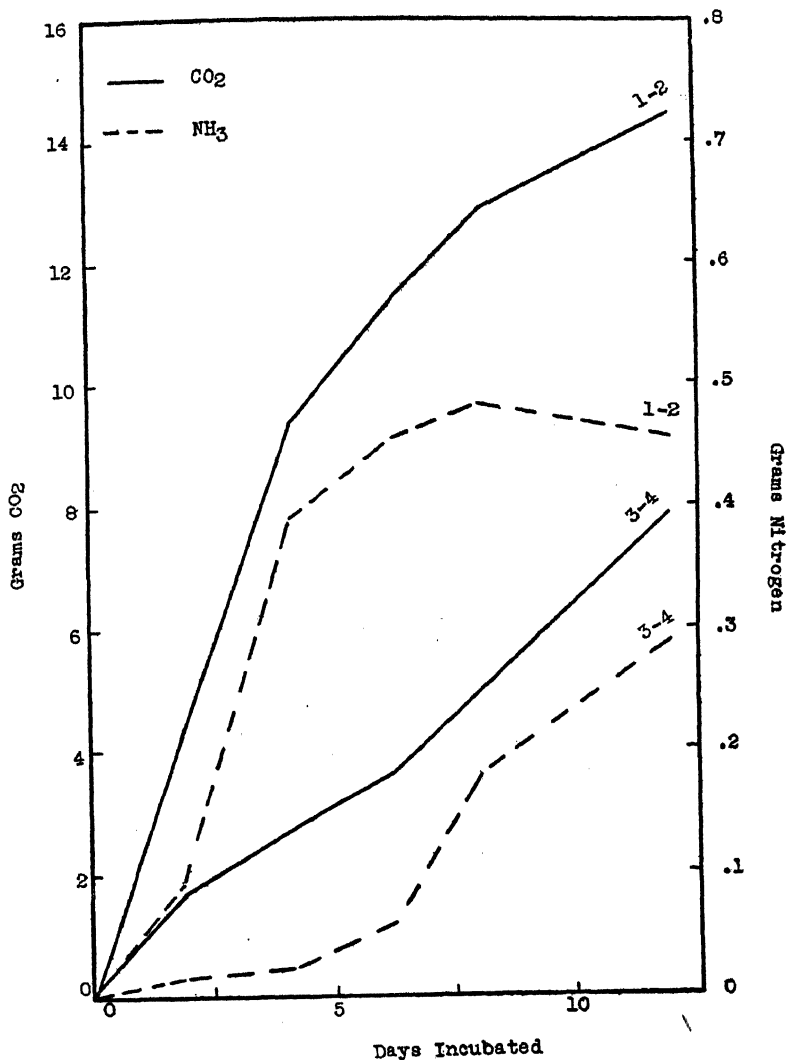


FIG. 22. Rate of decomposition of cottonseed meal in soil, as shown by the evolution of CO<sub>2</sub> and accumulation of NH<sub>3</sub>; 1 and 2 were aerated continuously, 3 and 4 were aerated 30 minutes daily (from Gainey).

present and presence of soluble phosphates (table 37). The period of maximum formation and accumulation of ammonia from protein substances by pure cultures of various fungi was found<sup>67</sup> to depend on the type of organism used; *Monilia sitophila* reached its maximum in 3 to 4 days, *Mucor plumbeus* reached it in 6 to 10 days.

*Rate of ammonia formation by microorganisms and methods of determination.* In the decomposition of proteins by pure cultures of bacteria or fungi, as well as in the soil itself, the rate of ammonia accumulation is that of an autocatalytic chemical reaction.<sup>68</sup> The nature of the protein and the presence of non-nitrogenous organic matter influence greatly the rate of the reaction. Gainey<sup>69</sup> observed a remarkable similarity in the rates of formation of ammonia and carbon dioxide from dried blood and cottonseed meal. At first there was a rapid increase which soon reached a maximum and then decreased rapidly. Insufficient aeration and moisture resulted in a decrease in ammonia formation. Unfavorable conditions had a more detrimental effect on the latter than on CO<sub>2</sub> evolution (fig. 22). An interesting correlation was found<sup>70</sup> between the amounts of ammonia formed and the numbers of bacteria, as a result of addition of organic matter to the soil.

The literature on the subject of ammonia formation from the decomposition of nitrogenous organic substances added to the soil is very extensive. Unsuccessful attempts have been even made to determine the productive capacity of a soil by its ammonia producing power, as will be shown later.

Among the methods used for determining ammonia in the soil and in solution, only three need be mentioned: (1) the direct distillation of the soil or solution with magnesium oxide; (2) the aeration method; (3) the extraction of ammonia with KCl solution, followed by distilling the ammonia with magnesium oxide.

The first is more rapid, but may bring about the liberation of some ammonia from amides and perhaps from other simple nitrogenous substances. The aeration method can be used in determining ammonia in liquid culture, but requires a long time for a complete extraction of the ammonia from soils.<sup>71</sup> It consists in placing

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<sup>67</sup> Waksman, S. A. and Cook, R. C. *Soil Sci.*, 1: 375-384. 1916.

<sup>68</sup> Miyake, 1916 (p. 458); Waksman, S. A. *Jour. Bact.*, 3: 475-492. 1918.

<sup>69</sup> Gainey, 1919 (p. 610).

<sup>70</sup> Beckwith, T. D., Vass, A. F. and Robinson, R. H. *Ore. Agr. Exp. Sta. Bul.* 118. 1914.

<sup>71</sup> Potter, R. S. and Snyder, R. S. *Jour. Ind. Engin. Chem.*, 7: 221. 1915; Gibbs, W. M., Neidig, R. E. and Batchelor, H. W. *Soil Sci.*, 15: 260-268. 1923.

25 to 50 cc. of the culture or soil suspension in large heavy glass tubes or flasks, adding some heavy oil, 2 to 3 grams of NaCl and 2 grams of  $\text{Na}_2\text{CO}_3$ , then aerating for 2 to 3 hours. The ammonia is absorbed in a standard solution of sulfuric acid to which a proper indicator has been added (like sodium alizarine sulfonate). On placing the tubes in a water bath, at 55 to 60°C., the process is carried out more rapidly and completely. Where heat is used, there is, of course, always some danger of hydrolysis of undecomposed proteins or their derivatives.

The extraction of the ammonia from soil by a KCl or NaCl solution is based upon the fact that the ammonium base is replaced in its adsorbed condition in the soil by another base when added in excess to the soil. The process is usually carried out by extracting 25 grams of the soil successively with five to seven 100-cc. portions of approximately 4 per cent chloride solution (or until the filtrate gives no test for ammonia with Nessler's reagent). Peat soils should be extracted two or three times more. Alkaline soils should be first neutralized with ammonia-free hydrochloric acid. The combined filtrates are then distilled with MgO into standard 0.1N  $\text{H}_2\text{SO}_4$ . When distillation is finished, the carbon dioxide is removed from the distillate by boiling, before the acid is titrated back with a standard alkali.<sup>72</sup>

*Nitrogen transformation in the decomposition of organic matter in the soil.* When nitrogenous organic substances are added to the soil, a group of complex reactions will result as far as the nitrogen is concerned:

(1) The hydrolysis of the proteins into polypeptides and amino acids, with the liberation of some ammonia. (2) This is followed by the decomposition of the amino acids and other products of protein hydrolysis, with a further liberation of ammonia. (3) Synthesis of microbial protoplasm, which will lead to a storing away of a part or the whole of the ammonia nitrogen; the greater the quantity of available non-nitrogenous organic matter accompanying the nitrogenous substances, the greater will be the synthesis of microbial protoplasm, leading to a greater assimilation of the nitrogen and to a smaller accumulation of ammonia. (4) Various soil conditions, as well as differences in the composition of the nitrogenous and the accompanying non-nitrogenous organic substances, lead to the development of different microorganisms capable of decomposing the nitrogenous materials; the carbon-nitrogen metabolism of these microorganisms is different; this leads, therefore, to differences in the amounts of ammonia liberated in a free state.

These reactions result in a transformation of a larger or smaller part of the nitrogen of the organic complexes into ammonia, which either as such or after it has been oxidized to nitrate, is available as a source of nitrogen for the growth of cultivated plants. In view of the fact that the liberation of ammonia is of such great economic importance, numerous contributions have been made to the subject, known as "am-

<sup>72</sup> Tarassoff, B. Zhur. Opit. Agron., 15: 118-138. 1914; Bengtsson, N. Soil Sci., 18: 255-278. 1924.

monification." These studies were chiefly limited to adding about 1 gram of the nitrogenous organic material to 100 grams of soil, mixing, placing in tumblers, then bringing the moisture content of the soil to optimum (60 per cent saturation), incubating for 4 to 14 (usually 7) days, then measuring the amount of ammonia present in the soil by distilling with  $MgO$ .

It was found<sup>73</sup> that organic nitrogenous materials of different origin liberated ammonia with a different degree of rapidity as follows:

NATURE OF MATERIAL	AMMONIA FORMED, PER CENT OF NITROGEN ADDED	
	4 days	7 days
Blood.....	18.24	32.28
Tankage.....	32.35	38.52
Fish.....	49.07	55.39

The difference in rapidity of decomposition of the organic nitrogen compounds with the liberation of ammonia is due to the nature of the nitrogen complex,<sup>74</sup> accompanying non-nitrogenous substances and environmental conditions, which favor the development of specific organisms and, therefore, specific processes.

*Influence of non-nitrogenous organic matter upon the decomposition of nitrogenous compounds and upon the amounts of ammonia liberated.* Hirschler<sup>75</sup> was the first to point out that the decomposition of proteins by microorganisms is modified by the presence of carbohydrates which prevent the formation of aromatic products of putrefaction. Indol, phenol, and oxy-acids were not formed in the decomposition of proteins by bacteria when sucrose, starch, dextrin, glycerol or lactic acid were present; i.e., the presence of an available source of energy modified the decomposition processes. It was later<sup>76</sup> demonstrated conclusively that bacteria do not decompose large amounts of proteins in the presence of available carbohydrates; the amount of ammonia formed may also be greatly diminished. This is due to the fact that the organisms derive their energy preferably from carbohydrates and act upon the proteins only to an extent sufficient to obtain the nitrogen required for the syn-

<sup>73</sup> Lipman, J. G. et al. N. J. Agr. Exp. Sta. Bul. 246. 1912.

<sup>74</sup> Jodidi, S. L. Iowa Agr. Exp. Sta. Res. Bul. 9. 1912; Lathrop, E. C. Soil Sci., 1: 509-532. 1916.

<sup>75</sup> Hirschler, A. Ztschr. physiol. Chem., 10: 306-317. 1886.

<sup>76</sup> Kendall, A. I. Jour. Inf. Dis., 30: 211. 1922; 17: 442-453. 1915; Jour. Amer. Chem. Soc., 35: 1201-1249. 1913; 36: 1937-1962. 1914.

thesis of their protoplasm. Any ammonia that is produced, in this connection, may be reassimilated. In the absence of available carbohydrates, proteins are used also as sources of energy and large amounts of nitrogen are liberated as waste products in the form of ammonia. The presence of an available carbohydrate does not inhibit, but rather stimulates the multiplication of the bacteria; it lessens, however, the amount of protein to be utilized, and, therefore, the amount of ammonia accumulated. Doryland<sup>77</sup> explained this by the fact that the bacteria utilize the ammonia as a source of nitrogen and the carbohydrates as a source of energy.

Thus, in the presence of available carbohydrates, two factors are at work: (1) less of the protein is decomposed since the bacteria and fungi prefer the carbohydrate to the protein as a source of energy, (2) the ammonia that has been formed from the decomposition of the proteins may be reassimilated by the microorganisms which utilize the carbohydrate as a source of energy. These microbes are, therefore, competing with higher plants, for the available nitrogen compounds in the soil. As a result of these studies, Doryland defined ammonification as "an expression of an unbalanced ratio for microorganisms, in which the nitrogen is in excess of the energy-nitrogen ratio." If the available energy material is equal to or is in excess of the energy-nitrogen ratio required by the flora, the coefficient of ammonia formation tends to approach zero; it tends to approach a maximum, if the available energy material is less than the energy-nitrogen ratio. Depending on the proportion of energy material to nitrogenous substances, "beneficial" bacteria may become "harmful." This is brought out in table 38.

*Bac. subtilis* produced, in the absence of glucose, 1 mgm. of  $\text{NH}_3$  for every 49 mgm. casein decomposed. In the presence of glucose, 1874.1 mgm. of casein was decomposed; this should have produced 38.2 mgm.  $\text{NH}_3$ , whereas only 11.9 mgm. were found. The difference between the actual amount of ammonia present in the glucose medium and the amount that would have accumulated, had the glucose been left out, is 26.3 mgm.; this quantity of ammonia must have been assimilated by the bacteria. At the same time, 1934 mgm. of glucose has disappeared or about 13 mgm. of  $\text{NH}_3$  for every 1 gram of glucose. The amount of nitrogen utilization by *Bac. subtilis*, with casein as a source of nitrogen, was found to be considerably greater than the nitrogen assimilated by this organism from inorganic salts in synthetic media, with glucose as a source of energy. This is due not only to the actually greater assimilation of nitrogen, but also because

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<sup>77</sup> Doryland, C. J. T. N. D. Agr. Exp. Sta. Bul. 116. 1916.



the organisms had at their disposal the energy that was made available from that part of the casein which has undergone decomposition.<sup>78</sup>

The various other bacteria behaved in a similar manner, differing, however, not only in the absolute amount of ammonia liberated, but also in the ratio between the glucose consumed, casein decomposed and ammonia liberated. *Bac. mycoides* liberated not only the largest absolute amount of ammonia, but consumed a smaller amount of casein per

TABLE 38

*Influence of glucose on ammonia accumulation from casein in 400 cc. of synthetic solution in six days at room temperature*

ORGANISM		NH <sub>3</sub> ACCU- MULA- TED	DIFFER- ENCE DUE TO GLU- COSE	CASEIN CONSUMED		GLU- COSE CON- SUMED	BAC- TERIA
				Total	Per 1 mgm. of NH <sub>3</sub>		
		mgm.	mgm.	mgm.	mgm.	mgm.	millions
<i>B. subtilis</i> .....	Casein	43.0		2109.1	49.0		28.8
	Casein + glucose	11.9	31.1	1874.1		1934	42.6
<i>B. proteus</i> .....	Casein	13.6		2054.4	151.0		62.4
	Casein + glucose	2.6	11.0	1716.4		1771	67.7
<i>B. mycoides</i> .....	Casein	64.9		1459.0	22.4		50.2
	Casein + glucose	14.3	50.6	1001.0		1885	61.0
<i>B. megatherium</i> ...	Casein	20.8		2055.2	98.8		57.8
	Casein + glucose	10.5	10.3	2086.5		1864	87.9
<i>B. vulgatus</i> .....	Casein	32.0		1734.9	54.2		91.4
	Casein + glucose	16.7	15.3	1773.1		1988	111.2
<i>Sarcina lutea</i> .....	Casein	28.4		829.5	29.2		14.7
	Casein + glucose	9.2	19.2	900.0		1565	15.6

unit ammonia liberated. Glucose brought about in all cases an increase in the numbers of bacteria, but a decrease in the amount of casein decomposed.

<sup>78</sup> It can be easily demonstrated in the case of fungi that the nitrogen content of the organism is higher when the energy is obtained from proteins than from carbohydrates. Further information on the influence of the carbon source on nitrogen utilization by *Bac. subtilis* is found in a paper by Aubel, E. A. Compt. Rend. Acad. Sci., 171: 478-479. 1920.

A small amount of sugar (0.05 per cent) may even have a stimulating effect on the formation of ammonia from casein by causing an increase in the numbers of bacteria. After the sugar has all been decomposed, the increased numbers of bacteria will bring about a greater consumption of energy and, therefore, a greater decomposition of the protein and liberation of ammonia (table 39). A change from a depressing to a stimulating effect by the addition of a small amount of available carbohydrate upon the accumulation of ammonia from dried blood is

TABLE 39

*Influence of various concentrations of glucose on the formation of ammonia from casein*<sup>79</sup>

	INCUBATION	C*		C + 0.05 PER CENT G		C + 0.1 PER CENT G		C + 0.2 PER CENT G	
		NH <sub>3</sub>	Bact.	NH <sub>3</sub>	Bact.	NH <sub>3</sub>	Bact.	NH <sub>3</sub>	Bact.
	days	mgm.	mill.	mgm.	mill.	mgm.	mill.	mgm.	mill.
<i>B. subtilis</i> .....	2	15.7	2.9	13.0	4.6	12.0	4.9	13.2	4.0
	4	25.1	11.4	20.0	22.8	15.9	30.9	13.0	33.0
	6	49.5	49.0	55.0	54.7	48.7	54.3	17.0	59.0
<i>B. proteus</i> .....	2	13.8	5.1	10.9	6.0	11.8	6.8	10.9	6.9
	4	20.2	21.1	22.0	39.2	13.7	48.3	15.0	50.1
	6	30.3	86.6	32.0	80.1	30.1	89.1	15.7	92.2
<i>B. mycoides</i> .....	2	22.6	4.7	14.8	4.4	13.9	3.8	14.0	4.8
	4	64.0	25.9	20.1	32.1	14.4	33.0	15.0	37.0
	6	69.6	63.2	60.0	65.9	52.0	66.7	11.9	66.1
<i>Sarcina lutea</i> ....	2	12.9	1.9	11.2	2.0	11.9	2.8	10.8	3.1
	4	22.4	5.9	10.2	7.6	11.9	6.1	12.0	7.4
	6	26.1	7.9	20.0	19.0	11.3	10.7	11.0	8.9

\* C = casein, G = glucose; Bact. = bacteria in millions.

illustrated in fig. 23. In the presence of undecomposed organic matter, the soluble nitrogen salts are transformed into insoluble proteins; these compounds are decomposed later and make the nitrogen compounds available again.<sup>79</sup>

Since the formation of ammonia is a prerequisite to nitrate formation, one would expect from these results that an excess of available energy

<sup>79</sup> Gerlach and Deusch. Mitt. Kais. Wilh. Inst. Landw. Bromberg, 4: 259. 1912 (Centrbl. Bakt. II, 37: 296. 1913).

would repress nitrate formation in the soil. Nitrification was found<sup>80</sup> to be checked when the carbon-nitrogen ratio in the soil is 13-15 to 1, but not when the ratio is 11-11.6 to 1. When molasses was added to the soil, nitrification was stopped when the ratio was about 11:1, but was not injured when the ratio was less. However, the addition of carbon sources not readily available, such as butyric acid and alcohol, did not injure nitrification at a ratio of 14:1, but injured it at a higher ratio. This phenomenon is brought out clearly when cellulose is added to the soil. The organisms using the cellulose as a source of energy

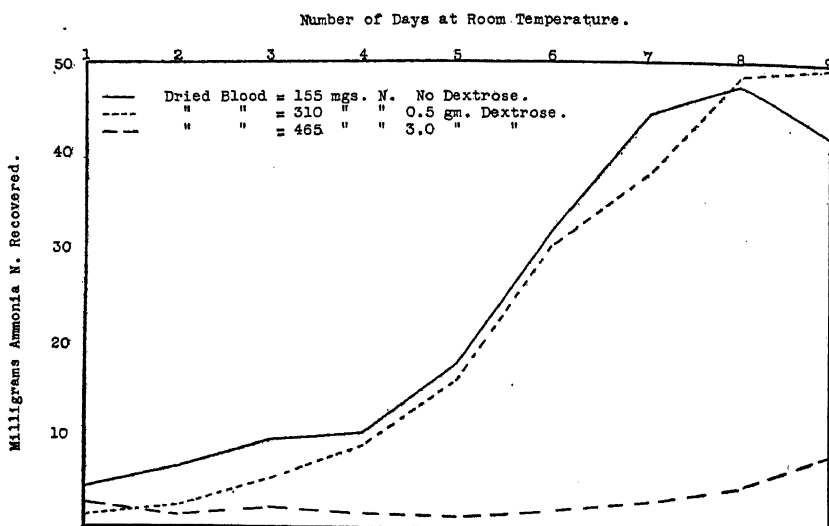


Fig. 23. Influence of glucose on ammonia accumulation from dried blood added to soil (after Lipman and associates).

assimilate the soil nitrates, without injuring, however, the activities of the nitrifying bacteria. Only after all the cellulose has decomposed, do nitrates begin to accumulate again, as shown in fig. 24.

As long as there is free available energy, in excess of the available nutrients, there will be only a minimum accumulation of available plant food. When the energy approaches exhaustion ammonia (or nitrate) begins to accumulate, as shown in table 40.<sup>81</sup> In due time, however, there is a narrowing of the energy-nitrogen ratio in the organic

<sup>80</sup> Clark, H. W. and Adams, G. O. Jour. Ind. Engin. Chem., 4: 272-274. 1912.

<sup>81</sup> Waksman, S. A. Jour. Amer. Chem. Soc., 39: 1503-1512. 1917.

material which undergoes decomposition, and an ultimate liberation of nitrogen in an available form will take place. Since the microorganisms

TABLE 40

*Influence of concentration of sugar upon the accumulation of ammonia from 2 per cent peptone solution by Asp. niger*

INCUBATION	SUGAR ADDED	NH <sub>4</sub> -N IN 100 CC.	WEIGHT OF MYCELIUM	SUGAR LEFT IN MEDIUM
days	per cent	mgm.	mgm.	
5	0	44.80	200	0
5	1	40.74	280	+
5	3	14.14	1,304	++
5	5	1.26	1,500	+++
5	20	0	1,620	+++++
15	0	73.08	360	0
15	1	50.68	930	0
15	3	36.54	3,270	0
15	5	33.04	5,220	+
15	20	0	11,210	++

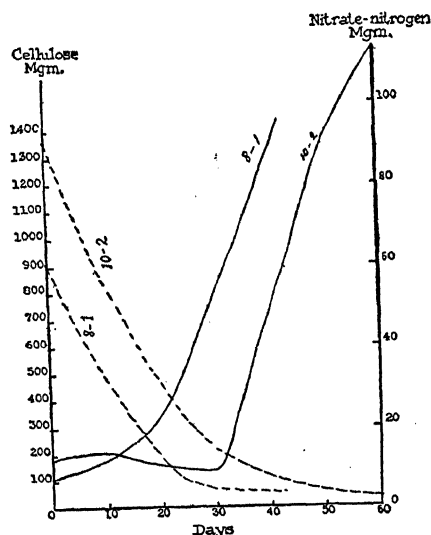


FIG. 24. Influence of cellulose on nitrate accumulation in the soil. cellulose; ——— nitrate nitrogen. 8-1 and 10-2 are the experiment numbers (from Anderson).

are unable to assimilate it, due to the absence of sufficient available energy material, it is left in the soil for the use of higher plants.

*Decomposition of organic substances of varying carbon-nitrogen ratio.* The nature and composition of the organic matter greatly influence its decomposition. The ratio between the carbon and nitrogen of the material used is of especial importance in this connection. The same is true of the nature of the non-nitrogenous organic materials introduced into the soil in addition to the nitrogenous substances. Table 41 has been compiled from the results of Lipman and associates,<sup>82</sup> who added different organic nitrogenous materials to 100 gram portions of soil; the moisture content was brought to an optimum and the soils incubated for 7 days, when the ammonia was determined by distilling with MgO. Rice flour and corn meal, with a wide carbon-nitrogen

TABLE 41

*Influence of carbohydrates upon the accumulation of ammonia from nitrogenous organic materials*

NITROGENOUS SUBSTANCE	TOTAL NITROGEN IN MATERIAL	AMMONIA FORMED, MILLIGRAMS			
		No carbo- hydrate	Glucose	Sucrose	Starch
<i>4 grams</i>	<i>mgm.</i>		<i>2 grams</i>	<i>2 grams</i>	<i>2 grams</i>
Rice flour.....	46.4	1.26	1.30	1.48	0.87
Corn meal.....	51.2	1.18	1.30	1.04	0.69
Wheat flour.....	94.8	5.14	3.66	5.84	1.56
Cowpea meal.....	156.8	50.88	31.71	28.57	23.70
Linseed meal.....	247.0	110.69	96.01	60.73	63.34
Soybean meal.....	245.6	129.64	108.03	94.88	54.36
Cottonseed meal.....	246.1	123.63	99.67	97.23	54.54

ratio, allowed no accumulation of ammonia at all either with or without any additional carbohydrate. The substances rich in nitrogen allowed an accumulation of almost 50 per cent of the nitrogen as ammonia, but this was considerably reduced when more available energy in the form of carbohydrates was added.

Table 42 illustrates the formation of ammonia from different organic materials when sufficient non-nitrogenous organic matter (starch) is added so as to introduce the same amount of fresh undecomposed organic matter. In these studies, the various amounts of the organic materials were added to 100 gram portions of soil and the ammonia determined after 9 days.<sup>83</sup> When the accumulation of ammonia from

<sup>82</sup> Lipman, J. G. et al. N. J. Agr. Exp. Sta. Bul. 247. 1912.

<sup>83</sup> Kelley, W. P. Hawaii Agr. Exp. Sta. Bul. 39. 1915.

the various nitrogenous substances is compared, it is found to be, with the exception of casein, in direct relation to the nitrogen content of the materials. When the same amount of nitrogen is added to the soil, the ammonia accumulated will depend upon the concentration of carbonaceous substances present. These may serve as sources of energy

TABLE 42

*Per cent of organic nitrogen transformed into ammonia in soil (Kelley)*

SOURCE OF NITROGEN	NITROGEN CONTENT	1 GRAM OF EACH ORGANIC MATERIAL ADDED TO 100 GRAMS SOIL	132.9 MGM. OF ORGANIC NITROGEN ADDED TO 100 GRAMS SOIL		132.9 MGM. ORGANIC NITROGEN PLUS ENOUGH STARCH TO MAKE EQUIVALENT AMOUNTS OF CARBON
		NH <sub>4</sub> -N	Aerobic conditions NH <sub>4</sub> -N	Anaerobic conditions NH <sub>4</sub> -N	NH <sub>4</sub> -N
	per cent	mgm.	mgm.	mgm.	mgm.
Casein.....	12.40	50.2	56.9	53.2	31.4
Dried blood.....	13.29	42.4	49.3	12.3	18.9
Soybean cake.....	8.28	40.9	48.7	14.0	34.1
Cottonseed meal.....	5.10	27.1	32.0	8.5	34.0
Linseed meal.....	5.00	26.0	34.6	6.9	34.1

TABLE 43

*Influence of composition of amino acid upon ammonia production by microorganisms*

AMINO ACID	C/N	ORGANISM	DRY GROWTH OF CELLS	NH <sub>4</sub> -N	GROWTH NH <sub>4</sub> -N
			mgm.	mgm.	
Glycocoll.....	1.7	<i>Trichoderma</i>	50	24.28	2.0
Glycocoll.....	1.7	<i>Actinomyces</i>	59	30.46	2.0
Alanine.....	2.57	<i>Trichoderma</i>	80	21.98	3.6
Alanine.....	2.57	<i>Actinomyces</i>	126	39.17	3.2
Glutamic acid.....	4.28	<i>Trichoderma</i>	218	29.12	7.5
Glutamic acid.....	4.28	<i>Actinomyces</i>	169	28.36	5.9
Glutamic acid.....	4.28	<i>Bact. fluorescens</i>	128	28.50	4.5

for the microorganisms, so that less of the protein is decomposed and more of the nitrogen is used up for the synthesis of microbial protoplasm. When enough starch is added to make the carbon concentration equal in all cases, the amount of ammonia accumulated will generally be constant. When the concentration of nitrogen is very high as in the case of dried

blood, the amount of starch added was very large; since this is a very readily available source of carbon, its rapid decomposition is accompanied by a greater disappearance of the ammonia nitrogen. Casein contains more nitrogen in comparison to carbon; therefore, the quantity of nitrogen liberated as the waste product (ammonia) from the casein is greater. When sufficient carbon is added to the casein, the amount of ammonia is the same as that of the cottonseed meal and linseed meal.

The rapidity of liberation of nitrogen in microbial cell substance as ammonia and its transformation into nitrate also depends upon the carbon-nitrogen ratio<sup>84</sup> of the material, as shown by Barthel:

CELL SUBSTANCE OF	NITROGEN CONTENT	NITROGEN ADDED TO 200 GRAMS OF SOIL	NITRATE FORMED IN 2 MONTHS
	<i>per cent</i>	<i>mgm.</i>	<i>Per cent of N added</i>
<i>Azotobacter</i> .....	1.63	3.3	0
<i>Bact. radicola</i> .....	3.90	7.8	34.1
<i>Urob. pasteurii</i> .....	11.41	22.8	60.7

According to Jodidi,<sup>85</sup> the formation of ammonia from various amino acids differs with the composition of the amino-acid molecule. In the case of glycocoll, 80 per cent of the nitrogen was transformed into ammonia, while in the case of leucine only 49 per cent nitrogen was changed to ammonia, under the same conditions. This difference was ascribed to the inert paraffin character of the comparatively long chain of the leucine molecule. However, the results presented in table 43 show that the greater the carbon content of the acid the more abundant is the growth of the organism and the less is the relative amount of nitrogen liberated as ammonia, i.e., the ammonia liberated from the decomposition of a definite amount of amino acid does not depend upon the absolute amount of nitrogen of the material decomposed, but upon the relative carbon-nitrogen content. The lower the ratio of carbon to nitrogen, the greater is the amount of ammonia liberated per unit of material decomposed.

*Carbon-nitrogen ratio of organic matter and growth of microorganisms.* Fungi, as a rule, can readily obtain their energy from carbohydrates and proteins. Actinomyces and heterotrophic bacteria prefer proteins,

<sup>84</sup> Heck, A. E. Soil Sci., 27: 1-48. 1929; Barthel, C. and Bengtsson, N. Proc. First. Intern. Congr. Soil. Sci., (1927), 3: 204-208. 1928.

<sup>85</sup> Jodidi, 1912 (p. 440).

peptones and certain peptides as sources of energy to carbohydrates, especially to the polysaccharides. This accounts for the difference in behavior of these organisms towards the various plant and animal residues and manures added to the soil. When ground alfalfa, which contains about 2.5 to 3.0 per cent nitrogen, is added to the soil, sterilized and inoculated with fungi, only a slight accumulation of ammonia takes place, although the alfalfa is rapidly decomposed, as indicated by the abundant  $\text{CO}_2$  production. This is due to the fact that fungi produce an abundant growth and use as much as 50 to 60 per cent of the carbon for structural purposes. Since fungi contain about 4 to 6 per cent

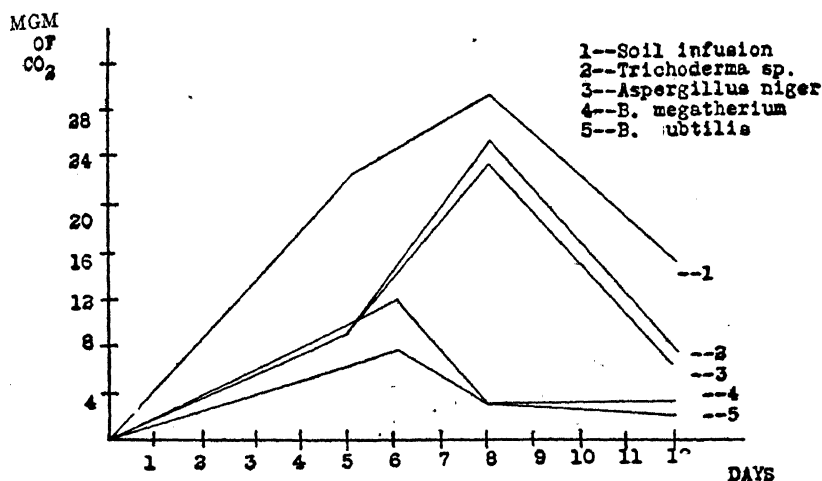


FIG. 25. Rate of decomposition of alfalfa meal by pure cultures of microorganisms and by the mixed soil flora, as indicated by the daily evolution of  $\text{CO}_2$  (from Neller).

nitrogen in their protoplasm, the nitrogen made available from the decomposition of the alfalfa may be just sufficient for the synthesis of the fungus protoplasm, without any excess left as ammonia.

When the same amount of alfalfa is added to soil, sterilized and then inoculated with actinomyces or bacteria, some ammonia will be readily formed. This is due to the fact that these organisms synthesize a considerably smaller amount of protoplasm than the fungi. The nitrogen content of those organisms is higher than that of fungi, viz., 4 to 12 per cent, but, because of the considerably lower carbon assimilation, a great deal of the nitrogen is liberated as ammonia. In a series of detailed



studies on the decomposition of organic matter by pure cultures of microorganisms, Neller<sup>86</sup> (figs. 25, 26) found that, under sterile conditions, fungi bring about a much greater evolution of  $\text{CO}_2$  than bacteria, the action of pure cultures of fungi approaching that of the complex mixture of organisms found in a soil suspension. The amount of ammonia liberated by the fungi, particularly by the rapidly growing forms (*Asp. niger*), was negligible in comparison with that liberated by the bacteria. In 12 days, *Trichoderma* and *Asp. niger* liberated, from 1 per cent alfalfa, about 21 per cent of the carbon as  $\text{CO}_2$  and a mere trace of the nitrogen as ammonia. *Bac. subtilis* liberated, in 8 days, from 2 per cent alfalfa, only 8.9 per cent of the carbon as  $\text{CO}_2$ , but 10.4 per cent of the nitrogen

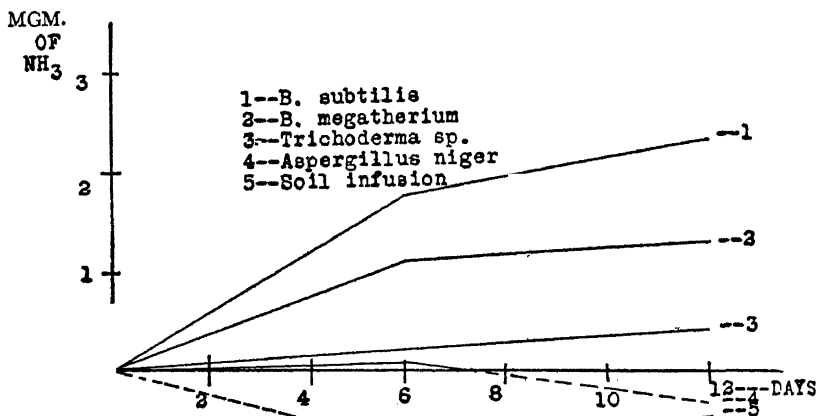


FIG. 26. Rate of decomposition of alfalfa meal by pure cultures of microorganisms and by the mixed soil flora, as indicated by the accumulation of ammonia (from Neller).

was changed to ammonia. This tends to demonstrate that, with a substance that has a C:N ratio of 16 (alfalfa meal), fungi require all the nitrogen for synthetic purposes, while bacteria can liberate as ammonia an amount of nitrogen proportional to the amount of carbon decomposed. Since fungi produced as great an amount of  $\text{CO}_2$  as the complex soil suspension, Neller suggested that the fungi can be looked upon as organisms active in normal soil. However, if fungi were the predominating or the only organisms in the soil, this state of affairs could hardly be considered beneficial to higher plants.

The rapid decomposition of organic matter by fungi, with a lack of

<sup>86</sup> Neller, J. R. Soil Sci., 5: 225-241. 1918.

ammonia accumulation, is true only of substances with a relatively wide carbon-nitrogen ratio, such as alfalfa meal; these substances are usually acted upon first of all by fungi and only later by bacteria. The fungi rapidly break down the complex carbohydrates and cause a narrowing of the carbon-nitrogen ratio. This leads to an abundant liberation of  $\text{CO}_2$  but not of ammonia, since the proteins are decomposed to a limited extent and all the ammonia is reassimilated. The synthesized fungus mycelium is richer in nitrogen than the alfalfa and fungi are efficient utilizers of available energy. The bacteria first of all break down the proteins and liberate large quantities of ammonia as a waste product. Due to the limited utilization of the cellulose and to the low nitrogen content of the bacteria, only a small amount of the ammonia is reassimilated. This is the reason why bacteria produce small amounts of  $\text{CO}_2$ , while considerable ammonia may be liberated. The quantities of ammonia and of nitrate produced and accumulated in the soil depend, therefore, upon the carbon-nitrogen ratio of the organic matter added.

*Summary.* The whole process of protein transformation and protein synthesis in the soil is very complex and is constantly in a dynamic condition. The net result is fertility or infertility, depending on which set of factors predominates in the soil at any one time. A mere determination of the amount of ammonia formed after adding to the soil a definite quantity of a certain organic fertilizer or plant material, like dried blood or cottonseed meal, cannot solve the question of availability of the nitrogen in the particular material; it does not indicate the amount of intermediate compounds formed in the decomposition of the organic matter in the soil, and the character of the action of these compounds on plant growth.

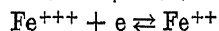
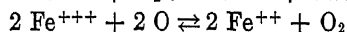
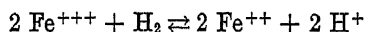
## CHAPTER XVIII

### OXIDATION PROCESSES IN THE SOIL—NITRATE FORMATION

*Oxidation-reduction phenomena.* Oxidation-reduction processes have commonly been interpreted in terms of oxygen; *oxidation* designated a process whereby oxygen was added to a substance or hydrogen was removed; *reduction* was applied to reactions involving the removal of oxygen or addition of hydrogen. However, in certain processes of oxidation-reduction no oxygen or hydrogen participate, as in the following reaction:



Clark and associates<sup>1</sup> were, therefore, led to consider these processes in the light of addition or withdrawal of electrons. Taking the oxidation and reduction of iron, the following reactions may be given:



When the reaction proceeds from left to right, reduction is taking place; when it proceeds from right to left, oxidation is taking place. In the presence of methylene blue, nitrate or other substances capable of acting as hydrogen acceptors, oxidation may take place even in the absence of atmospheric oxygen; this enables certain bacteria to live anaerobically. Processes of oxidation can thus be considered either as (1) aerobic processes, in which atmospheric oxygen acts as the hydrogen acceptor or the oxidizing agent and (2) anaerobic processes, in which organic or inorganic compounds act as the hydrogen acceptors, both processes resulting in the liberation of energy.

In addition to the action of unactivated molecular oxygen as a hydrogen acceptor, there are at least three ways in which activation of oxygen can occur: (1), by means of iron, which acts as an oxygen carrier;<sup>2</sup> (2) by

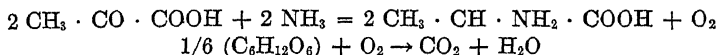
<sup>1</sup> Clark, W. M., et al. Public Health Reports, **38**: 443, 666, 933, 1669; **39**: 381, 804. 1924.

<sup>2</sup> Warburg, O. Biochem. Ztschr., **152**: 479-494. 1924.

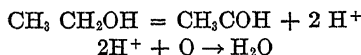
the action of the sulfhydryl group;<sup>3</sup> (3) by substances which are capable of peroxide formation.<sup>4</sup>

The oxidation-reduction character of bacteriological media exerts an important influence on the growth and behavior of the organisms towards atmospheric oxygen. A knowledge of the oxidation-reduction potential of the media is important not only in obtaining the optimum growth of an organism, but also for the purpose of using the medium for counting organisms, by the plate or dilution methods. The lengthened lag-phase of growth represents the time necessary for an organism to change the potential of the medium to a more favorable condition.<sup>5</sup>

The oxygen which may be liberated in synthetic microbial processes is consumed directly for the liberation of energy, as in the formation of amino acids from organic acids and ammonia, which is the first step in protein synthesis by heterotrophic microorganisms:



The oxygen in the  $\text{CO}_2$  molecule that has thus become liberated is not derived from free gas but from a molecule capable of reduction by glucose, a phenomenon distinctly different from that involving oxygen occurring in an external medium.<sup>6</sup> In considering oxidation as the activation of hydrogen, atomic oxygen will be formed if the hydrogen of water is activated. In the presence of substances, such as methylene blue or nitrates, which readily absorb hydrogen, oxidation becomes possible as a result of the reduction of the active hydrogen. For example, acetic acid bacteria oxidize alcohol in the absence of oxygen but in the presence of methylene blue, the latter acting as a hydrogen acceptor.



The rôle of oxygen consists in binding the hydrogen and its place can be taken by other hydrogen acceptors. The acetaldehyde is changed

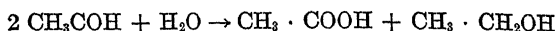
<sup>3</sup> Hopkins, F. G. *Biochem. Jour.*, **19**: 787. 1925; *Jour. Biol. Chem.*, **84**: 269. 1929.

<sup>4</sup> Quastel, J. H. *Biochem. Jour.*, **20**: 166-194. 1926.

<sup>5</sup> Allyn, W. P. and Baldwin, I. L. *Jour. Bact.*, **20**: 417-439. 1930.

<sup>6</sup> Aubel, E. and Wurmser, R. *Compt. Rend. Acad. Sci.*, **179**: 848-851. 1924. A detailed review of the processes of energy transformation involved in oxidation-reduction is given by R. Wurmser. *Oxydations et reductions*. Presses Universitaires. Paris. 1930.

by the Cannizzaro reaction to alcohol and acetic acid, this process being accompanied by the liberation of energy:



The phenomena of oxidation and reduction by microorganisms are brought about in most instances by means of specific enzymes of the *oxidase-peroxidase* nature on the one hand and *reductase* or *perhydridase* nature on the other.

The oxidation-reduction intensities of biological systems can be determined colorimetrically by the use of indigo-sulfonate or other appropriate indicators, or by means of an electrode of a noble metal. By immersing two electrodes in two liquids of different oxidation potential connected with a siphon, a current is formed and oxidation will occur in the solution about one electrode and reduction in the solution about the other. The intensity factor can thus be measured. There is a relation between the hydrogen-ion concentration and oxidation-reduction potential of the cell.<sup>7</sup>

*Oxidation processes in the soil.* Liebig<sup>8</sup> recognized that proper oxidation is essential for the decomposition of plant and animal residues added to the soil. Mulder<sup>9</sup> called attention to the fact that oxidation as well as reduction processes take place in the soil at the same time, with a certain equilibrium established between the two. Oxidation processes usually lead to the complete decomposition of organic substances in the soil. Among the other important processes of oxidation, it is sufficient to mention the oxidation of ammonium salts to nitrites and of the latter to nitrates, the oxidation of elementary sulfur and sulfur compounds to sulfates, and the oxidation of hydrogen, methane and other substances produced by processes of incomplete oxidation or reduction. Oxidation processes may be looked upon as beneficial in the soil. Reduction processes may often become harmful, since, with incomplete oxidation, substances may be formed which are directly injurious to plant growth. It is sufficient to indicate that the reduced forms of nitrates (nitrites), of sulfates (sulfites) and of phosphates (phosphites) are toxic to plant growth.

<sup>7</sup> Gillespie, K. J. *Soil Sci.*, 9: 199-216. 1920; Remesow, P. *Ztschr. Pflanz. Düng. Bodenk.*, 15: 34-44. 1929. Needham, J. and D. M. *Proc. Roy. Soc. B*, 98: 259-286. 1925.

<sup>8</sup> Liebig, J. *Chemie in ihrer Anwendung auf Agrikultur*. 4 Aufl., 1842.

<sup>9</sup> Mulder. *Die Chemie der Ackerkrume*. 1. 1863, Tr. by Müller. Berlin.

Dehérain and Demoussy<sup>10</sup> demonstrated that, in the process of oxidation of organic matter, oxygen is always taken up and carbon dioxide is set free. They distinguished between microbial and chemical oxidation. Microbial oxidation is most active at normal temperatures and is favored by increased temperatures, 65° being the maximum; the quantities of oxygen absorbed and CO<sub>2</sub> produced are found to differ with soil type, moisture, aeration. The volume of CO<sub>2</sub> produced is usually smaller than that of oxygen absorbed. Chemical oxidation is low at normal temperatures and increases with temperature elevation, reaching a maximum at 110°. The CO<sub>2</sub> produced chemically often exceeds the oxygen absorbed. Russell<sup>11</sup> found that oxidation increases with an increase in the fertility of the soil. The rate of oxidation increases with temperature, amount of water (up to a certain optimum) and amount of CaCO<sub>3</sub>. Heating of soil to 100°C. or treatment with volatile antiseptics, which are subsequently removed, bring about a great increase in the oxygen absorbed.

Schreiner and Sullivan<sup>12</sup> used a solution of aloin (0.125 per cent) for the study of soil oxidation, the change in color being taken as an index of oxidation. As determined by this method, oxidation in soil was found to be non-enzymatic in nature and was considered a result of interaction between inorganic constituents and certain types of organic matter. The addition of salts of Mn, Fe, Al, Ca and Mg increased this type of oxidation. Factors decreasing oxidation in soils were found to bring about conditions which decrease soil productivity and vice versa.

*Source of nitrates in the soil.* The decomposition of proteins and of other nitrogenous organic substances leads to the formation and often the accumulation of ammonia in the soil. Under favorable conditions, this is rapidly oxidized to nitrites and then to nitrates. Under certain conditions, when the nitrifying bacteria are killed, as in the partial sterilization of soil, or when conditions do not favor nitrification, as with excessive soil acidity, ammonia may accumulate in the soil.

The intense accumulation of nitrates in places where large quantities

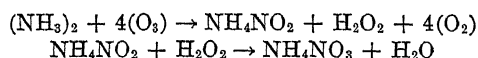
<sup>10</sup> Dehérain, P. P. and Demoussy, E. Ann. Agron., 22: 305-337. 1896.

<sup>11</sup> Russell, E. J. Jour. Agr. Sci., 1: 261-279. 1905; Darbishire and Russell, 1908 (p. 728).

<sup>12</sup> Schreiner, O. and Sullivan, M. X. Bur. Soils, U. S. Dept. Agr. Bul. 73. 1910; Schreiner, O. and Reed, H. S. Ibid., Bul. 56. 1909.

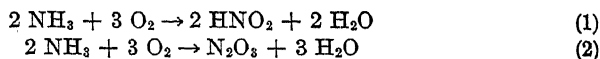
of organic matter are allowed to decompose was explained by the chemists of the earlier part of the nineteenth century to be a result of chemical processes. Davy<sup>13</sup> expressed the opinion that nitrates are formed at the expense of the ammonia of the soil and of atmospheric oxygen; Liebig demonstrated that atmospheric nitrogen takes no part at all in the process of nitrification.

The oxidation of ammonia to nitrate can be accomplished by chemical means, especially at high temperatures and in the presence of catalysts,<sup>14</sup> as in the case of the electrolytic oxidation of ammonia in the presence of copper oxyhydrate.<sup>15</sup> Ammonia can be oxidized to nitrate, to a limited extent, in an atmosphere saturated with ammonia and in the presence of ferric hydrate.<sup>16</sup> Ammonia is also oxidized to nitrite by ultra-violet radiation.<sup>17</sup> According to Weith and Weber,<sup>18</sup> hydrogen peroxide and ammonia react with each other giving rise to nitrous acid. The interaction between ozone and ammonia to give ammonium nitrate has been known for several decades:



The quantities of nitrite and nitrate formed by chemical agencies are insignificant and of little importance in the soil. The biological nature of nitrification, as established by Schloesing and Müntz, Warington<sup>19</sup> and others, is most important.

*Mechanism of ammonia oxidation.* Various reactions have been suggested to explain the mechanism of oxidation of ammonia to nitrite by the nitrite forming bacteria. The following two reactions are most probable:



<sup>13</sup> Davy, H. Elements of agricultural chemistry. 1814, p. 308.

<sup>14</sup> Sestini, F. Landw. Vers. Sta., 60: 103-112. 1904; Mooser, W. Landw. Vers. Sta., 75: 53-106. 1911.

<sup>15</sup> Traube, W. and Biltz, A. Ber. deut. chem. Gesell., 39: 166-178. 1906.

<sup>16</sup> Russell, E. J. and Smith, N. Jour. Agr. Sci., 1: 444-453. 1906.

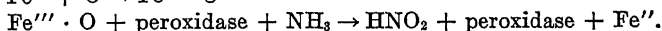
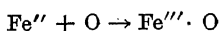
<sup>17</sup> Berthelot, D. and Gaudechon, H. Compt. Rend. Acad. Sci., 152: 522-524. 1911.

<sup>18</sup> Weith, W. and Weber, A. Ber. deut. chem. Gesell., 7: 1745-1749. 1874.

<sup>19</sup> Schloesing, Th. and Müntz, A. Compt. Rend. Acad. Sci., 84: 301. 1877; 85: 1018. 1877; 86: 892. 1878; 89: 891, 1074. 1879; Warington, R. Jour. Chem. Soc., 33: 44-51. 1878; 35: 429-456.

Godlewski<sup>20</sup> found that the ratio between the oxygen consumed in the process and the nitrogen changed from the ammonia to the nitrite stage is 3, or  $R = \frac{O_2}{N_2} = 3$ . However, Schloesing<sup>21</sup> recorded previously that this ratio varies from 4.57 to 5.57. The difference between these results may be due to the fact that Schloesing made his determinations in the soil as a medium while Godlewski carried out his studies in solution. In the first instance, some of the oxygen was no doubt consumed for other oxidation processes, in addition to nitrate formation. Meyerhof<sup>22</sup> used the theoretical value of  $R = 3$  for calculating from the amount of oxygen consumed, the quantity of nitrogen oxidized from ammonia to nitrite. In a careful study of the gaseous exchange in the reaction of oxidation of ammonia to nitrite, Bonazzi<sup>23</sup> demonstrated that the average value of  $R$  is  $2.89 \pm 0.08$ , which is very close to 3.0, thus justifying the above equations.

The presence of carbonate is essential both as a source of  $CO_2$  (although the organism can also use free  $CO_2$  present in the atmosphere), and for keeping the medium properly buffered, preventing it from becoming acid. Nitrites are formed also in an atmosphere free from  $CO_2$  (but containing  $CO_2$  in the medium), although at a slower rate. The cells of the organism are strongly catalytic and are capable of liberating oxygen from a peroxide. Ferric hydrate has a stimulating effect upon the oxidation of ammonia to nitrate.<sup>24</sup> Since iron was found in active cultures partly in a ferrous state, Bonazzi suggested that it acts as a carrier of oxygen, thereby hastening the oxidation process; iron fulfills in these cultures the functions of the peroxide, due to its mechanism of auto-oxidation resulting in a combination with oxygen, while the cells liberate the oxygen thus bound.



In 100 cc. of medium and at 35°, 20 mgm. of nitrogen in the form of ammonium sulfate can be oxidized in 6 hours. Nitrite formation comes to a standstill, when the solution contains about 1.5 to 2.0 per cent  $NO_2$ . The concentration of the substrate which is most favorable for the

<sup>20</sup> Godlewski, K. *Centrbl. Bakt.* II, **2**: 458-462. 1896.

<sup>21</sup> Schloesing, Th. *Compt. Rend. Acad. Sci.*, **109**: 423-428, 883-887. 1885.

<sup>22</sup> Meyerhof, O. *Pflüg. Arch. Ges. Physiol.*, **164**: 353-427; **165**: 229-284. 1916; **166**: 240-280. 1917.

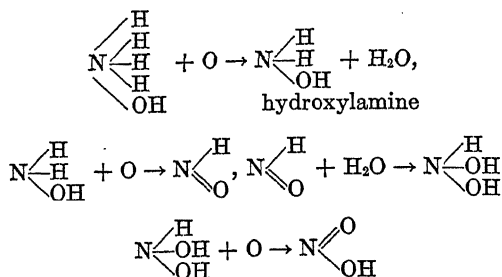
<sup>23</sup> Bonazzi, A. *Jour. Bact.*, **8**: 343-363. 1923.

<sup>24</sup> Ashby, S. F. *Jour. Agr. Sci.*, **2**: 52-67. 1907.

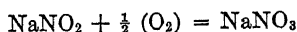


oxidation process lies at 0.005 M  $\text{NH}_4$ , while with a tenth molar solution of ammonia oxidation is nil.

The more recent theories tend to indicate that the oxidation of ammonia to nitrous acid goes through the hydroxylamine and hyponitrous acid stages.<sup>25</sup>



*Mechanism of nitrite oxidation.* The oxidation of nitrite to nitrate takes place according to the following reaction:



This was demonstrated to hold true by measuring the nitrite and oxygen consumption. With optimum concentration of the nutrients and proper aeration of the culture, the nitrate forming organisms, in liquid culture, may oxidize 4 to 5 grams  $\text{NaNO}_2$  per liter in 24 hours.

According to Miyake,<sup>26</sup> nitrification in the soil obeys the law of autocatalysis; i.e., the reaction is at first slow, then becomes more rapid and finally comes to a standstill, as a result of accumulation of nitrates. The relation between nitrite oxidation and concentration of substrate is given in figure 27, which shows a rapid increase in oxidation with an increase in nitrite concentration up to 0.05 per cent. An optimum is reached with 0.1 per cent of substrate, with a slow decrease to 0.3 per cent. This is followed by a gradual drop, so that in a 4 per cent solution of nitrite, oxidation is only 26 per cent of the optimum. This injury is caused also by other salts in similar osmotic concentrations. A decrease in the concentration of oxygen lessens both growth and respiration, so that at  $\frac{1}{10}$  atmosphere pressure, respiration is decreased by 66 per cent. This injury is reversible. Growth may be even more injuriously affected than respiration. For growth and respiration defi-

<sup>25</sup> Kluyver, A. J. and Donker, H. J. L. *Chem. d. Zelle u. Gewebe.*, **13**: 134-190. 1926.

<sup>26</sup> Miyake, K. *Soil Sci.*, **2**: 481-492. 1916; Miyake, K. and Soma, S. *Jour. Biochem. Tokio*, **1**: 123-9. 1922.

nite concentrations of  $O_2$ ,  $NO_2$ ,  $NO_3$ ,  $OH^-$  and traces of  $H_2CO_3$  are required. The other salts and nutrients play only the part of buffering agents.

Winogradsky observed the interesting phenomenon that ammonium salts injuriously affect the growth of nitrate bacteria. This seemed rather strange in view of the fact that the nitrate bacteria are active side by side with the nitrite bacteria which use the ammonium salt as a source of energy. It was then<sup>27</sup> suggested that the two processes

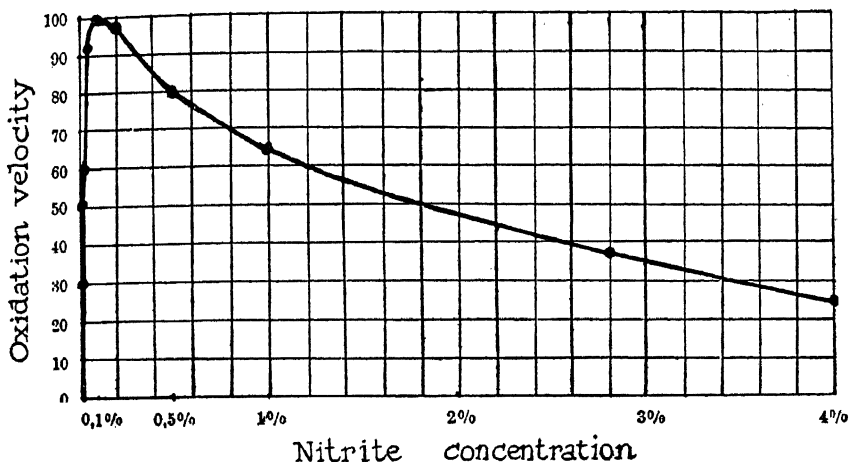


FIG. 27. Influence of nitrite concentration upon the oxidation velocity of nitrate forming bacteria (from Meyerhof).

follow in two successive periods in the soil, nitrate formation beginning only after all the ammonium salt is converted into nitrite. On decreasing the amount of  $Na_2CO_3$ , which would lead to a lower alkalinity, Boulanger and Massol<sup>28</sup> found that the injurious effect of ammonium salt is less and concluded, therefore, that the growth of nitrate bacteria is not injured by the salt but by free ammonia. This was confirmed by Meyerhof who established that the injurious influence of ammonia and its derivatives (aliphatic amines) consists in the penetration of the base into the cell (which does not take place in the case of ammonium

<sup>27</sup> Omeliansky, W. L. *Centrbl. Bakt.* II, 5: 473-490. 1899; see Bonazzi, A. *Jour. Bact.*, 6: 479-499. 1921.

<sup>28</sup> Boulanger, E. and Massol, L. *Ann. Inst. Pasteur.*, 18: 181-196. 1904; Löhnis, F. and Blobel, S. *Fühlings landw. Ztg.*, 57: 385-402. 1908.

salt) and in a specific action of the  $\text{NH}_3$  and  $\text{NH}_2$  groups. Lipoid insoluble amines, like the diamines, are not injurious. The injurious effect of amines and cations depends upon their ability to penetrate into the cell and upon the reaction of the media; respiration is usually less affected than growth. The intermediary products of the oxidation of sulfur (hyposulfite) produce a decided injury upon the process of nitrate formation in soil; the nitrifying bacteria as such are not injured, since the process is resumed as soon as these intermediary products have disappeared.<sup>29</sup>

*Nitrate formation from inorganic salts and from organic nitrogenous compounds.* Schloesing compared the formation of nitrates from various ammonium salts added to the soil and found that the following relative amounts of nitrogen (in milligrams) are nitrified per day:

$$\text{NH}_4\text{Cl} - 3.4, (\text{NH}_4)_2\text{SO}_4 - 9.0, (\text{NH}_4)_2\text{CO}_3 - 4.0$$

Ammonium salts of organic acids are also nitrified rapidly.

It was thought at first that organic matter can be nitrified directly. However, Müntz<sup>30</sup> has shown that organic matter has to be decomposed first and ammonia liberated, before nitrates can be formed. Omeliansky later obtained negative results also for urea, asparagine, methylamine, dimethylamine and egg-albumin, so that he concluded that all forms of organic nitrogen have to be transformed first into ammonia before they can be nitrified. The same was found to hold true for calcium cyanamide.<sup>31</sup> When the processes of nitrate formation from ammonium salts and from amino acids are compared, the latter is found to take place more slowly.<sup>32</sup> This is probably due to the fact that the amino-acids have to be changed first to ammonia and also to the fact that some of the nitrogen is stored away in the microbial cells which use the carbon of the amino compounds as a source of energy.

According to Barthel and Bengtsson,<sup>33</sup> only the ammonia (and the urea, which is rapidly transformed into ammonia) of stable manure is readily nitrified; the other part of the nitrogen, present in the manure

<sup>29</sup> Guittonneau, G. Compt. Rend. Acad. Sci., 184: 898, 1518-1520. 1929.

<sup>30</sup> Müntz, A. Compt. Rend. Acad. Sci., 110: 1206-1209. 1890.

<sup>31</sup> de Grazia, S. Staz. sper. agr. ital. Modena, 41: 241-257. 1908.

<sup>32</sup> Batham, H. N. Soil Sci., 20: 337-351. 1925.

<sup>33</sup> Barthel, Chr. and Bengtsson, N. Medd. Centralanst. Försök. Jordbruk-somradet, No. 211, 1920; also 1926 (p. 388).

in the form of complex proteins, nitrifies only very slowly since it has to be decomposed first by various microorganisms, with the liberation of ammonia. Nitrite bacteria find conditions in stable manure very favorable for their development so long as aeration is favorable and the manure is not saturated with urine.<sup>34</sup> Deep layers as well as com-

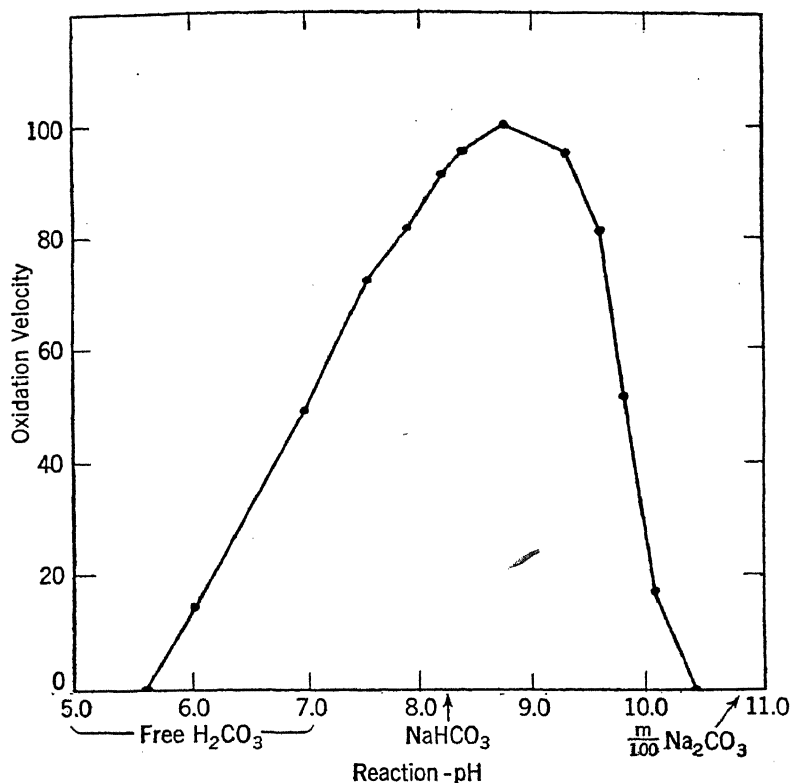


FIG. 28. Influence of reaction upon nitrate formation by bacteria (after Meyerhof).

pact manure contain only few nitrifying bacteria, since conditions are not very favorable for their development, under anaerobic or semianaerobic conditions. The nitrite bacteria cannot develop in urine, probably because of the presence of some injurious substances and the high concentration of the soluble organic matter; they develop readily in

<sup>34</sup> Niklewski, B. Centrbl. Bakt. II, 26: 388-442. 1910; Bull. Soc. Chim. biol., 5: 491-500. 1923.

the solid portion of the manure. The free ammonia, especially in a medium of a high alkalinity, is also injurious to the nitrate-forming bacteria.

*Influence of reaction on nitrate formation.* The optimum reaction for the respiration of the nitrite-forming bacteria was found<sup>35</sup> to be at pH 8.4 to 8.8, with limiting reactions at pH 7.6 and 9.3. The optimum reaction for the respiration of the nitrate-forming bacteria was found to be at pH 8.3 to 9.3 and the limits at pH 5.6 and 10.3, as shown in

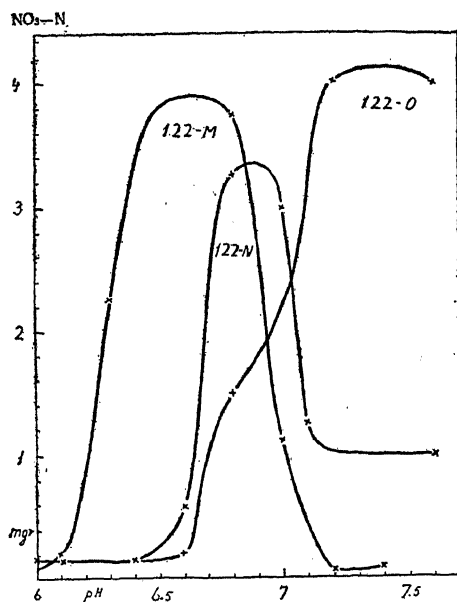


FIG. 29. Influence of hydrogen-ion concentration upon the oxidation of ammonium sulfate by 3 more or less pure strains of bacteria (after Gaarder and Hagem).

figure 28. The presence of  $\text{NaHCO}_3$ , which acts as a buffer at pH 8.4, is, therefore, beneficial to the activities of these organisms. This is true, however, only for the respiration of the organisms but not for their growth.

The optimum reaction for the growth of the nitrite bacteria is at pH 7.8, with a minimum at pH 7.0 and maximum at pH 8.6; for nitrate bacteria the optimum is at 7.1 with limiting reactions at 6.5 and 7.8. The kind of buffer used is of importance. The limiting acid reaction

<sup>35</sup> Meyerhof, 1916 (p. 457).

for the growth of nitrifying bacteria in soil has been found<sup>36</sup> to be at pH 3.9 to 4.5, depending on the origin of these bacteria and the reaction of the soil from which they were obtained; those isolated from acid soils are better adapted to acid conditions (fig. 29). The limiting alkali reaction was found to be at pH 8.9 to 9.0; by gradual adaptation, the organisms can be made to grow at reactions beyond their acid and alkaline range so that nitrate formation was even obtained at pH 13.0<sup>37</sup>

When ammonium sulfate is used as a source of nitrogen for nitrate formation and the reaction of the soil is acid to begin with, there will be an increase in acidity in the absence of sufficient buffer or base, as a result of formation of nitric acid from the oxidation of the ammonia and the accumulation of the residual sulfuric acid. Nitrate accumulation will proceed until the reaction of the soil has reached a pH of about 4.0. The amount of nitrate formed under these conditions depends upon the initial reaction of the soil and its buffer and base content; the higher the buffer and base content of the soil, the larger will be the amount of nitrate formed for a certain change of reaction.<sup>38</sup> The continuous use of ammonium sulfate as a fertilizer without the addition of lime will, therefore, lead to a gradual increase in soil acidity. However, nitrates may be found even in very acid soils. This was explained by Hall and associates<sup>39</sup> as due to the fact that, under acid conditions, nitrate formation takes place in films surrounding the small isolated particles of  $\text{CaCO}_3$ . The addition of  $\text{CaCO}_3$  has, therefore, a decided stimulating effect on nitrate formation, particularly in acid soils.<sup>40</sup> In alkaline soils which are deficient in organic matter,  $\text{CaCO}_3$  may have the opposite effect since it tends to liberate from ammonium salts free ammonia, which retards nitrification.

Lime does not stimulate the activities of the nitrifying bacteria<sup>41</sup>;

<sup>36</sup> Gaarder and Hagem, 1920-1923 (p. 76); Meddel. 11, Vestland Forst. Forsksta. Bergen. 1928.

<sup>37</sup> Meek, C. S. and Lipman, C. B. Jour. Gen. Physiol., 5: 195-204. 1922.

<sup>38</sup> Barthel and Bengtsson, 1920 (p. 460); Waksman, S. A. Soil Sci., 15: 241-260. 1923.

<sup>39</sup> Hall, A. D., Miller, N. H. J. and Gimingham, C. T. Proc. Roy. Soc. B, 80: 196-212. 1908.

<sup>40</sup> Fischer, H. Landw. Jahrb. 41: 755-822. 1911; Vogel, J. Centrbl. Bakt. II, 32: 169-179. 1911; Lemmermann, O., Fischer, H. and Husek, B. Landw. Vers. Sta., 70: 317-342. 1909; Fred, E. B. Centrbl. Bakt. II, 39: 455-468. 1913; Miller, F. Ztschr. Gärungsphysiol., 4: 194-206. 1914; White, J. W. Ann. Rpt. Penn. Agr. Exp. Sta. 1913-14, 70-84.

<sup>41</sup> Stephenson, R. E. Iowa Agr. Exp. Sta. Res. Bul. 58. 1920; Temple, J. C. Ga. Agr. Exp. Sta. Bul. 103. 1914.

it serves as a base for neutralizing the acid formed from the oxidation of the ammonium salt. Nitrate formation takes place readily in peat and muck soils,<sup>42</sup> if the reaction is not too acid and if the soil is properly inoculated with the organisms. Acid peat soils do not generally offer a favorable medium for the development of the nitrate forming bacteria; when lime is added, these organisms become very active, leading often to a rapid diminution of available nitrogen,<sup>43</sup> as shown in table 44.<sup>43</sup>

*Influence of organic matter upon nitrate formation.* Small amounts of soluble organic matter were found to retard the activities of nitrite

TABLE 44

*Influence of application of lime upon nitrogen content and nitrification of a muck soil*

APPLICATION OF LIME	NITROGEN CONTENT OF SOIL	NITRATE FORMATION		REACTION	
		Original soil	Incubated soil	Original soil	Incubated soil
	<i>per cent</i>	<i>parts per million</i>	<i>parts per million</i>	<i>pH</i>	<i>pH</i>
None.....	1.120	28	114	3.67	3.56
Different forms of lime, 1 ton...	1.203	38	142	4.15	3.80
Different forms of lime, 2 tons..	1.151	37	162	4.37	4.24
Different forms of lime, 3 tons..	1.108	35	178	5.02	4.80
Different forms of lime, 4 tons..	1.054	37	205	5.46	5.24
Fertilizer.....	1.114	30	59	3.88	3.48
Fertilizer + limestone, 1 ton....	1.165	35	151	4.06	3.85
Fertilizer + limestone, 2 tons...	1.133	35	130	4.70	4.32
Fertilizer + limestone, 3 tons...	1.016	40	189	5.40	4.93
Fertilizer + limestone, 4 tons...	1.046	35	173	6.13	5.44

and nitrate forming bacteria (table 45). Glucose, which is so important for the activities of the majority of microorganisms, is injurious in concentrations of 0.025 to 0.05 per cent. In the soil, however, the organisms can stand high concentrations of organic matter.<sup>44</sup> Müntz and Lainé<sup>45</sup> concluded that organic matter in the soil may even be distinctly favorable to the activities of the nitrifying organisms. The greater the or-

<sup>42</sup> Arnd, Th. Centrbl. Bakt. II, 45: 554-574. 1916; Landw. Jahrb., 51: 297-328. 1918.

<sup>43</sup> Arnd, Th. Landw. Jahrb., 47: 372-442. 1914; 49: 191-213. 1916; Tiulin, A. F. Trans. Inst. Fertil. No. 26, 1925. Moskau; Willis, L. G. Tech. Bul. 24, N. C. Agr. Exp. Sta. 1923.

<sup>44</sup> Stevens, F. L. and Withers, W. A. Centrbl. Bakt. II, 27: 169-186. 1910.

<sup>45</sup> Müntz, A. and Lainé, E. Compt. Rend. Acad. Sci., 142: 430-435. 1906.

ganic content of the soil, the more abundant is its bacterial flora and the more rapid will be the process of nitrification taking place. It was later found<sup>46</sup> that, even in solution, impure cultures may be favored by soil extracts. According to Barthel,<sup>47</sup> the easily soluble organic substances must be mineralized in the soil before nitrate formation takes place, if no injurious effect is to occur. Difficultly soluble organic substances have little effect on the process.

*Influence of salts upon nitrate formation.* Most inorganic alkali salts have only a slightly injurious effect upon nitrate-formation, usually only at a concentration greater than 0.3 *N*; alkali earths are more injurious, 0.1 *N* causing an injury of 60 per cent. The injurious effect of cations of alkali salts depends to a large extent on the OH concentration. Nitrification takes place in solution in the presence of 10,000

TABLE 45

*Influence of organic matter upon the growth of nitrite and nitrate-forming bacteria*

SUBSTANCE	NITRITE ORGANISM		NITRATE ORGANISM	
	Growth checked	Growth stopped	Growth checked	Growth stopped
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Glucose.....	0.025	0.05	0.05	0.2
Peptone.....	0.025	0.2	0.08	1.25
Asparagine.....	0.025	0.3	0.05	0.5
Glycerol.....	More than 0.2	....	0.05	4.0
Urea.....	More than 0.2	....	0.5	4.0
Sodium acetate....	0.5	More than 1.5	1.5	3.0
Ammonia.....	.....	....	0.0005	0.015

parts per million of NaCl, but not in higher concentrations; Na<sub>2</sub>SO<sub>4</sub> was not injurious even in concentrations of 30,000 parts per million; Na<sub>2</sub>CO<sub>3</sub> was found to be most injurious.<sup>48</sup> The ratio of the calcium to magnesium is not of great importance to the activities of the nitrate-forming bacteria, but the total concentration of magnesium in solution

<sup>46</sup> Coleman, L. C. Centrbl. Bakt. II, 20: 401-420, 484-514. 1908. Mazé. Compt. Rend. Acad. Sci., 152: 1625. 1911; Fremlin. Jour. Hyg., 14: 149. 1914; Wright, 1916 (p. 485); Barthel. Centrbl. Bakt. II, 49: 382. 1919; Greaves and Carter. Jour. Agr. Res., 6: 889. 1916; Makrinow, J. Centrbl. Bakt. II, 24: 415. 1919; Löhnis and Green, 1914 (p. 509).

<sup>47</sup> Barthel, C. Ztschr. Gärungsphysiol., 4: 11-48. 1914.

<sup>48</sup> Lipman, C. B. Centrbl. Bakt. II, 33: 305-313. 1912.



and its relation to the concentration of the other constituents is of significance.<sup>49</sup>

Chlorides, nitrates, sulfates and carbonates of Na, K, Ca, Mg, Mn and Fe exert a toxic effect upon nitrate-formation in the soil, depending on the specific salt and not on the electro-negative ion.<sup>50</sup> The quantity of a salt which can be applied to a soil without decreasing the nitrate nitrogen accumulation varies with the nature of the salt. Those compounds which become toxic in lower concentrations are not necessarily most toxic in higher concentrations, as the toxicity of some salts increases more rapidly than the toxicity of others. The common alkali salts are highly toxic, including  $\text{CaCl}_2$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{Na}_2\text{CO}_3$ , and the less common  $\text{Ca}(\text{NO}_3)_2$ .

It has been claimed that manganese and arsenic<sup>51</sup> exert a stimulating effect upon nitrate formation. Montanari<sup>52</sup> could not confirm this so far as arsenic is concerned. Heavy metals inhibit nitrate formation according to their protein-precipitating properties, mercury and silver salts being most injurious. Copper, zinc, iron and lead may exert a stimulating effect.<sup>53</sup> Ashby<sup>54</sup> found that, in the presence of iron hydroxide, nitrification takes place even in the absence of carbonates; the catalytic effect of iron is very important in the growth and respiration of the organisms. The injurious influence of dicyanodiamide upon the activities of the nitrate forming bacteria is illustrated in fig. 30.

*Influence of soil gases upon nitrate formation.* A liberal supply of oxygen is favorable to nitrate formation.<sup>55</sup> The mere stirring of the soil was found to stimulate the process; this stimulating effect was believed to be due to better aeration (fig. 31). It is known, however, that nitrate formation takes place as rapidly in compact clay soils as in

<sup>49</sup> Kelley, W. P. Jour. Agr. Res., 7: 417-437. 1916; Centrbl. Bakt. II, 42: 577-582. 1914; Lipman, C. B. and Burgess, P. S. Plant World, 17: 295. 1914; Centrbl. Bakt. II, 41: 430-444. 1913.

<sup>50</sup> Greaves, J. B., Carter, E. G. and Goldthorpe, H. C. Jour. Agr. Res., 16: 107-135. 1919.

<sup>51</sup> Olaru, D. A. Rôle du manganèse en agriculture. Baillères, Paris. 1920; Greaves, J. E. Centrbl. Bakt. II, 39: 542-560. 1913; Biochem. Bul. 3: 2. 1913.

<sup>52</sup> Montanari, C. Staz. sper. agr. ital. Modena, 50: 69-72. 1917.

<sup>53</sup> Lipman, C. B. and Burgess, P. S. Univ. Cal. Publ. Agr. Sci., 1: 127-139. 1914.

<sup>54</sup> Ashby, 1907 (p. 457).

<sup>55</sup> Warington, R. Exp. Sta. Rec., 3: 894-903. 1892; King, F. H. and Whitson, A. R. Wisconsin Agr. Exp. Sta. Bul. 85. 1901; Bul. 93. 1902.

coarser grained soils, when the available water is the same in both cases.<sup>56</sup> This would tend to indicate that the amount of oxygen necessary for nitrate formation need not be abundant, so long as it is sufficient for the normal respiration of the organisms and the moisture supply is favorable. The optimum concentration of oxygen for nitrate formation was found<sup>57</sup> to be 35 per cent. Similar observations were made for

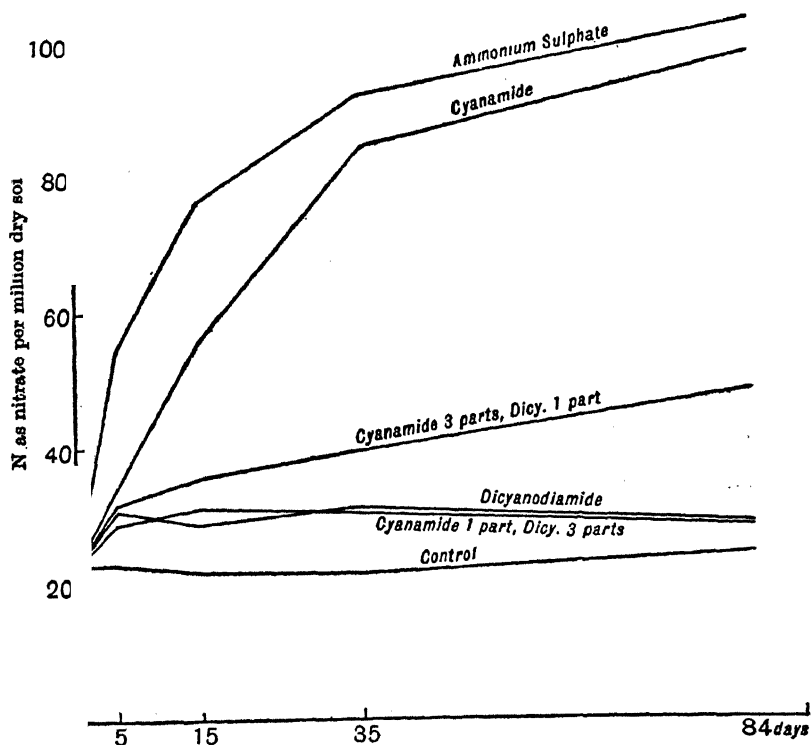


Fig. 30. Influence of dicyanodiamide upon nitrate production in the soil (from Cowie).

the influence of  $\text{CO}_2$  concentration.<sup>58</sup> Some believed that a supply of this gas is very important both for the nitrite and nitrate forming or-

<sup>56</sup> Schloesing, Th., Jr. *Compt. Rend. Acad. Sci.*, **125**: 824-827. 1897; Fischer, H. *Landw. Jahrb.*, **41**: 755-822. 1911; Gainey, P. and Metzler, L. F. *Jour. Agr. Res.*, **11**: 43-64. 1917.

<sup>57</sup> Plummer, J. K. N. Y. (Cornell) Univ. Agr. Exp. Sta. Bul. 384. 1916.

<sup>58</sup> Coleman, L. C. *Centrbl. Bakt.* II, **20**: 401-420, 484-514. 1908.

ganisms; others<sup>59</sup> found that  $\text{CO}_2$ , above a certain concentration, has no effect on nitrate formation in the soil. In view of the fact that the  $\text{CO}_2$  is used by the bacteria for the building up of their cells chemosynthetically, its presence is necessary for growth. But since the organisms produce a limited amount of growth, only small amounts of  $\text{CO}_2$  are required even for the maximum nitrification. Larger concentrations seem to act merely as an inert gas. In general, while only small amounts of  $\text{CO}_2$  are required, an excess of oxygen is essential; a lack of this gas produces anaerobic conditions which lead to nitrate reduction until all the nitrates are destroyed.

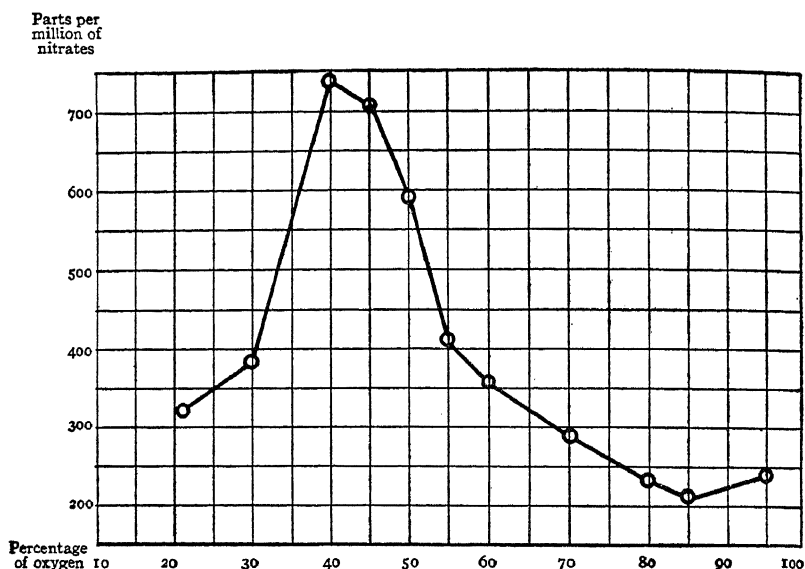


FIG. 31. Influence of oxygen tension upon nitrate formation in the soil (from Plummer).

One-tenth of a per cent of toluol and less than one per cent of  $\text{CS}_2$  do not exert any appreciable effect upon nitrate formation.<sup>60</sup> Larger quantities (5 to 10 times) exert a temporary retarding effect. When these substances are used for partial sterilization of soil nitrate-forming bacteria are killed, and it takes a long time before the soil becomes inoculated again.

<sup>59</sup> Owen, W. L. Georgia Agr. Exp. Sta. Bul. 81. 1908.

<sup>60</sup> Gainey, P. L. Centrbl. Bakt. II, 39: 584-595. 1914.

*Nitrate formation in solution and in soil.* Stevens and Withers<sup>61</sup> were the first to call attention to the fact that nitrate formation in solution inoculated with a certain amount of soil is not the same as nitrate formation in the soil itself. Nitrates are formed in the upper layers of soil, 90 per cent of the process being carried out in the upper 40 to 50 cm. not only in soils of humid regions,<sup>62</sup> but in saline soils as well.<sup>63</sup> This is due to the need of oxygen for the activities of the organisms. Even nitrate formation in solution is greatly stimulated by aeration.<sup>64</sup>

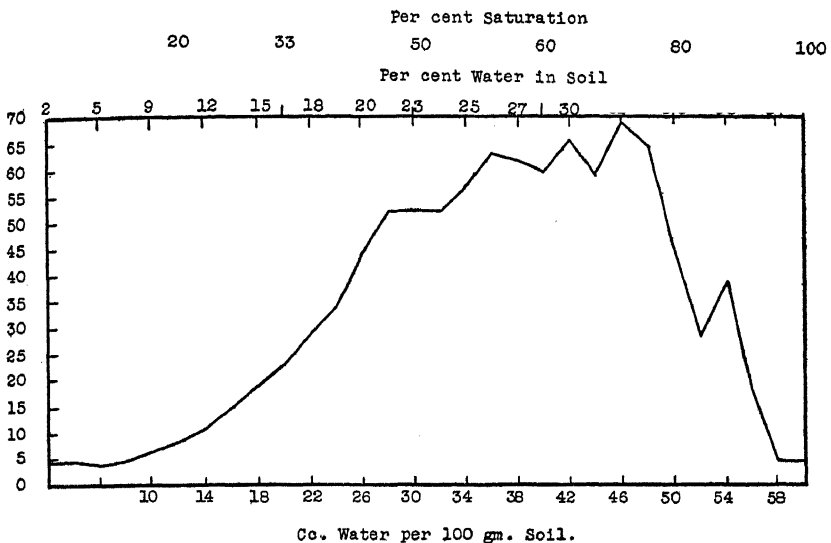


FIG. 32. Influence of moisture content upon nitrate production in the soil (from Gainey).

Dehérain<sup>65</sup> found that when the moisture content of the soil is 5 per cent the process of nitrate formation is very slight, but it becomes appreciable with 10 per cent moisture and reaches a maximum with

<sup>61</sup> Stevens, F. L. and Withers, W. A. *Centrbl. Bakt.* II, **23**: 355-373. 1909; **34**: 187-203. 1912.

<sup>62</sup> Koch, A. *Jour. Landw.*, **59**: 293-315. 1911; McBeth, I. G., and Smith, N. R. *Centrbl. Bakt.* II, **40**: 24-51. 1914.

<sup>63</sup> Bobko, E. B. et al. *Omsk.* 1928.

<sup>64</sup> Barthel, Chr. *Centrbl. Bakt.* II, **25**: 108-125. 1909.

<sup>65</sup> Dehérain, P. P. *Traité de Chimie agricole*, p. 586. 1902.

15 to 20 per cent. Schloesing and Müntz<sup>66</sup> reported that nitrate formation in soil is at a maximum with the highest moisture content which will not saturate the soil. When the soil approaches the saturation point, the process of nitrate formation is greatly reduced and may disappear completely<sup>67</sup> (fig. 32). The nature of the nitrogen source was found to be important in this connection. Abundant nitrate formation and even extensive accumulation may take place in semi-arid soils.<sup>68</sup> The amount and application of irrigation water has an appreciable influence upon the process of nitrate formation in these soils.

Air drying has a favorable effect upon the formation of nitrates in the soil; this effect is noticeable even after spreading out the soil for twenty-four hours and then remoistening.<sup>69</sup> Freezing of the soil in winter was also found to improve the nitrifying power of the soil.<sup>70</sup> According to Müntz and Gaudechon,<sup>71</sup> maximum nitrifying activities take place in spring (March 28 to April 25).

Conditions which tend to promote nitrate formation in the soil are: (1) temperature of 27.5°C., (2) an abundant supply of air (oxygen), (3) proper moisture supply, (4) a favorable reaction (pH greater than 4.6), (5) presence of carbonates or other buffering agents, and (6) absence of large quantities of soluble organic matter in the soil. The nature of the crop grown also influences the nitrate content of the soil. Fig. 77 (p. 756) brings out the effect of fallow upon the nitrate content of the soil at different seasons of the year.<sup>72</sup>

*Oxidation of minerals in soil.* When elementary sulfur is added to the soil, it is oxidized to a limited extent chemically and to a much greater extent biologically. A large number of organisms seem to be capable, in the presence of various organic substances, of oxidizing small amounts

<sup>66</sup> Schloesing, Th. and Müntz, A. Compt. Rend. Acad. Sci., **89**: 1075. 1879.

<sup>67</sup> Traaen, A. E. Centrbl. Bakt. II, **45**: 119-135. 1916.

<sup>68</sup> Stewart, R. Centrbl. Bakt. II, **36**: 477-490. 1913; Sackett, W. G. Colo. Agr. Exp. Sta. Bul. 193. 1914; Science, **42**: 452. 1914; Headden, W. P. Jour. Ind. Engin. Chem., **6**: 586-590. 1914; Col. Agr. Exp. Sta. Bul. 155, 160, 178, 179, 183, 184, 186, 193; Kelley, 1916 (p. 466); Stewart, R. and Greaves, J. E. Centrbl. Bakt. II, **34**: 115-147. 1912.

<sup>69</sup> Buddin, W. Jour. Agr. Sci., **6**: 452-455. 1914.

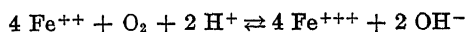
<sup>70</sup> Lyon, T. L. and Bizzell, J. A. N. Y. (Cornell) Univ. Agr. Exp. Sta. Mem. 1. 1913.

<sup>71</sup> Müntz, A. and Gaudechon, H. Compt. Rend. Acad. Sci., **154**: 163-8. 1912.

<sup>72</sup> See also Withers, W. A. and Fraps, G. S. Jour. Amer. Chem. Soc., **23**: 318-326. 1901; **28**: 213-233. 1906; Lipman, C. B. Jour. Ind. Engin. Chem., **9**: 189. 1917; Cal. Agr. Exp. Sta. Bul. 260. 1915; Lipman, J. G., Brown, P. E. and Owen, I. L. N. J. Agr. Exp. Sta., 31st Ann. Rept., 152-155. 1910.

of sulfur, with the formation of various compounds. Certain specific groups of bacteria seem to be most active in the process, since these organisms utilize the sulfur as a source of energy. This is true also of sulfides; the speed of oxidation of these depends on their solubility. Hydrogen sulfide and alkali sulfides are oxidized very readily and rapidly; alkali earth sulfides are oxidized more slowly; while the biological oxidation of iron sulfide (iron pyrites) has not yet been demonstrated.

The oxidation of ferrous ions to ferric ions is carried out in nature by the oxygen of the air:



The reaction of equilibrium depends entirely upon the pH of the medium and the oxygen pressure; in other words, the relationship between the activities of the ferrous and ferric ions depends upon the hydrogen ions and oxygen concentration. If the oxygen pressure is increased or the hydrogen-ion concentration is decreased or both are taking place, iron will be oxidized.<sup>73</sup> This results in an accumulation of precipitated iron. However, in the presence of organic forms of iron, oxidation may take place without precipitation. The oxidation reaction may take place as a result of strictly chemical changes; it may also be an indirect result of the modification of environment by the development of various microorganisms. In addition to these phenomena, the oxidation of ferrous iron is carried out by certain specific groups of bacteria, namely "iron bacteria," which utilize the energy liberated in the process for the chemosynthetic assimilation of CO<sub>2</sub> (p. 89).

A detailed study of the oxidation and reduction of arsenic compounds by microorganisms has been made by Van Zyl.<sup>74</sup>

*Oxidation of organic compounds in soil.* Attention has been called previously to the fact that various organic compounds are formed in the soil as a result of the activities of microorganisms. These may become toxic to the growth of higher plants unless further oxidized. Conditions favoring oxidation processes stimulate the decomposition of these substances and make conditions in the soil more favorable for the growth of higher plants. Various oxidation processes are essential for the liberation of a sufficient amount of energy for the activities of micro-

<sup>73</sup> Starkey, R. L. and Halvorson, H. O. *Soil Sci.* **24**: 381-402. 1927.

<sup>74</sup> Van Zyl, J. P. *Union S. Africa Dept. Agr. Repts. Div. Vet. Ed. and Res.* 9-10: 727-808. 1923 (*Exp. Sta. Reed*, **82**: 178).

organisms. The maximum energy is liberated by organic substances only when they are completely oxidized. Oxidation of amino acids and of purine bases are important soil processes, leading to the formation of ammonia.

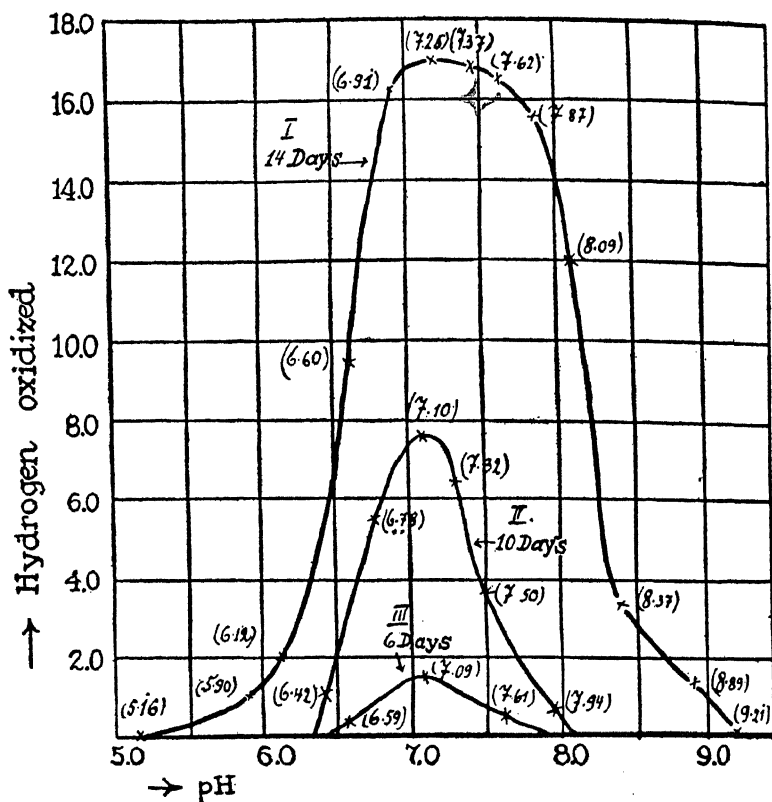
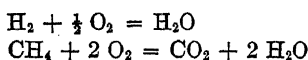


FIG. 33. Reaction of medium and oxidation of hydrogen by bacteria (from Ruhland).

Iron plays an important part as a catalytic agent in the oxidation of various substances. In the auto-oxidation of cysteine, an intermediate cysteine-iron complex is formed which is auto-oxidizable; this process is inhibited by HCN.<sup>75</sup>

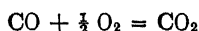
<sup>75</sup> Warburg, O. and Sakuma, S. *Pflüg. arch. ges. Physiol.*, **200**: 203. 1923; Sakuma, S. *Biochem. Ztschr.*, **142**: 68-78. 1923; Harrison, D. G. *Biochem. Jour.*, **18**: 1009-1022. 1924.

Among the gases produced in the anaerobic decomposition of organic matter, hydrogen and methane (in addition to  $\text{CO}_2$ ) occupy a prominent place. These gases are oxidized, under aerobic conditions, by certain specific bacteria (p. 93), according to the following reactions:

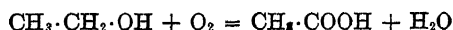


The bacteria are capable of utilizing the energy liberated from the oxidation of these materials for their activities and the chemosynthetic assimilation of carbon from  $\text{CO}_2$ ; they are, however, facultative autotrophic and are also capable of living heterotrophically in the absence of the specific sources of energy and in the presence of organic matter. The influence of reaction upon the oxidation of hydrogen is brought out in fig. 33.

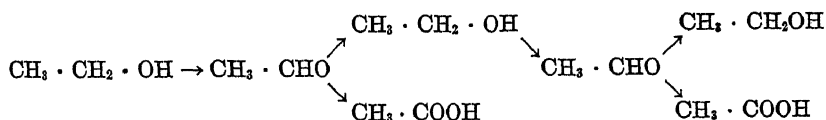
Carbon monoxide is also oxidized by bacteria, the energy liberated being utilized by the organisms.



In addition to these processes, a number of other oxidations are known to be carried out by soil organisms. It is sufficient to cite the oxidation of alcohol to acetic acid:



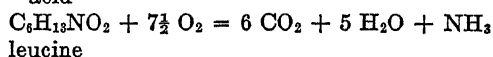
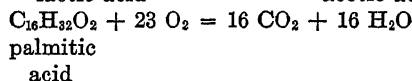
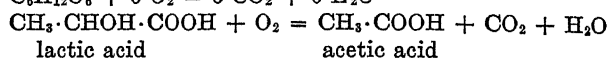
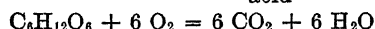
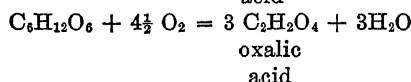
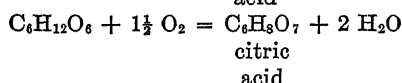
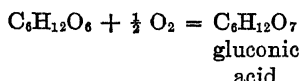
It has been suggested<sup>76</sup> that the ethyl alcohol is first oxidized to acetaldehyde, the latter being either oxidized further to acetic acid or used for the synthesis of microbial protoplasm:



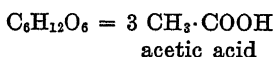
The numerous oxidations of sugars, organic acids, of proteins and of protein decomposition products are carried out by a number of soil fungi and bacteria, according to the following reactions:

<sup>76</sup>Neuberg, C. and Windisch, F. *Die Naturwiss.*, **13**: 993-996. 1925; *Biochem. Ztschr.*, **166**: 454-481. 1925.

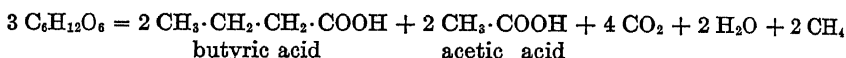




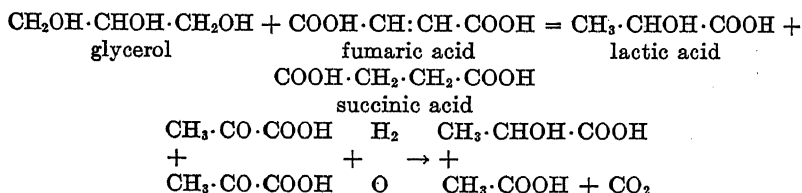
The processes known as fermentations, involving the anaerobic utilization of energy by processes of oxidation-reduction, stand in a class by themselves. Some are not accompanied by the liberation of  $\text{CO}_2$ .



In other processes,  $\text{CO}_2$  as well as other gases may be produced.



Simultaneous oxidation and reduction take place in the presence of a hydrogen donator and a hydrogen acceptor, as in the case of glycerol and fumaric acid or two molecules of pyruvic acid (by the Cannizzaro reaction).<sup>77</sup>



In the process involving the reduction of nitrate to gaseous nitrogen, nitrate acts as the hydrogen acceptor, as shown later (p. 478).

<sup>77</sup> Quastel, H. et al. *Biochem. Jour.* 19: 304-317, 660-666. 1925.

## CHAPTER XIX

### REDUCTION PROCESSES IN SOIL—NITRATE REDUCTION

*Reduction processes in the soil.* Just as aerobic conditions in soil favor oxidation processes, so do anaerobic conditions (exclusion of free oxygen) favor processes of reduction. Either organic or inorganic compounds may be formed, as a result of these processes, depending upon the composition of the medium.<sup>1</sup> It is not necessary for the soil to be saturated with water for the conditions to be anaerobic. Winogradsky<sup>2</sup> demonstrated, by the development of anaerobic nitrogen-fixing bacteria, that when a soil contains water equivalent to only about 40 per cent of its moisture-holding capacity, anaerobic bacteria find conditions favorable for their development even at the very surface of the soil.

A soil possessing a reducing power will form naphthol-blue from a mixture of para-nitroso-dimethylaniline and  $\alpha$ -naphthol but will not readily oxidize easily oxidizable substances, such as aloin.<sup>3</sup> Other indicators, like p-nitromalachite green which is reduced to p-amino malachite green,<sup>4</sup> can also be used to determine the oxidation-reduction potential of the soil. Reduction phenomena are characterized by the reduction of inorganic salts rich in oxygen, especially nitrates and sulfates. In the absence of atmospheric oxygen, the organic matter of the soil is broken down with the formation of hydrogen which, in *statu nascendi*, brings about the reduction of salts rich in oxygen. Formation of  $H_2S$  is thus a secondary phenomenon. Under aerobic conditions, however, the formation of  $H_2S$  is primary, since it results from the decomposition of proteins. *Bact. coli*, for example, decomposes glucose under anaerobic conditions, with the formation of pyruvic acid and hydrogen.<sup>5</sup>

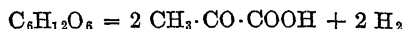
<sup>1</sup> Van Wolzogen Kühr, C. A. Arch. Suikerind, Nederlandsch. Indie, **23**: 501-511. 1915.

<sup>2</sup> Winogradsky, S. Compt. Rend. Acad. Sci., **179**: 861. 1924.

<sup>3</sup> Sullivan, M. X. Science, **39**: 958. 1914.

<sup>4</sup> Felton, L. D. Jour. Inf. Dis., **34**: 414-419. 1924.

<sup>5</sup> Aubel, E. and Salabartan, J. Compt. Rend. Acad. Sci., **180**: 1183-1186, 1784-1787. 1925.



Nitrates and sulfates can act as the hydrogen acceptors and are reduced to nitrites, sulfides, etc.

Reducing conditions in the soil have usually been recognized either by the absence of oxidation or by the presence of specific reducing substances, as ferrous carbonate; soils acquire these conditions when waterlogged for a few days. The  $\text{H}_2\text{S}$  formed from the reduction of sulfates combines with iron to form insoluble iron sulphide. The ferrous compounds themselves act as reducing agents. The very presence of these compounds indicates the intense reducing power of the soil.

Bacterial cultures themselves are normally reducing.<sup>6</sup> Processes of reduction require sources of energy to enable the organisms to carry on their activities. In most cases, however, these are obtained from various organic compounds.<sup>7</sup> The autotrophic bacteria use the energy obtained by chemosynthetic processes for the reduction of  $\text{CO}_2$ . Various organic compounds may be reduced under anaerobic conditions, especially in connection with oxidation of other substances which result in the liberation of energy. The reducing power of bacteria has commonly been determined by the use of certain organic substances, especially dyes, and of certain inorganic substances, such as nitrates and sulfates, acting as hydrogen acceptors. The hydrogen obtained from the decomposition of organic matter is used by the bacteria for the reduction of the dye<sup>8</sup> or the nitrate. In some cases aldehydes or purine bases (hypoxanthine, xanthine, adenine) are required as hydrogen donors.<sup>9</sup>

*Transformation of nitrates by microorganisms.* The disappearance of nitrates in soil as a result of activities of microorganisms may be due to three groups of phenomena: (1) direct utilization of nitrates by microorganisms as sources of nitrogen, in the presence of sufficient energy material, (2) reduction of nitrogen to nitrites and ammonia in the process of the nitrate assimilation, (3) utilization of nitrates as sources of oxygen (nitrates as hydrogen acceptors). In the last process oxygen is used by the organism for the oxidation of carbon compounds or inorganic substances, such as sulfur. The energy thus derived is used for the reduction of the nitrate to nitrite, free nitrogen gas, oxides of nitrogen or the ammonia stage. The formation of nitrogen gas from

<sup>6</sup> Rubner, M. Arch. Hyg., 16: 62. 1893.

<sup>7</sup> Beijerinck, M. W. Arch. Sci. Ex. Nat. Neerl. (II), 9: 131-157. 1904.

<sup>8</sup> Carapelle, E. Centrbl. Bakt. I, Orig., 47: 545-559. 1908.

<sup>9</sup> Dixon, M. and Thurlow, S. Biochem. Jour., 18: 989-992. 1924.

nitrate may be so rapid under favorable conditions that the gas can actually serve as a measure of the amount of nitrate reduced.

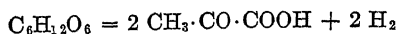
The disappearance of nitrates in the soil due to the various processes of nitrate reduction and nitrate assimilation has often been referred to as "denitrification." However, the reduction of nitrates to nitrites and ammonia as well as their assimilation by microorganisms does not involve any losses of nitrogen, but merely indicates that the nitrates are for the moment taken out of circulation and transferred into forms from which nitrate can be again produced. The nitrates may even completely disappear without involving any loss of nitrogen, as in the case of their assimilation by fungi and various bacteria in the presence of available energy.<sup>10</sup> The term *denitrification* (or complete denitrification) should designate the complete reduction of nitrates to atmospheric nitrogen and oxides of nitrogen, while the other processes involving disappearance of nitrates may be referred to as *nitrate reduction* and *nitrate assimilation*.

*Nitrate assimilation.* Large numbers of microorganisms, including bacteria, actinomyces, fungi and algae are capable of utilizing nitrates as a source of nitrogen. In the presence of a sufficient amount of available energy, the microorganisms rapidly assimilate the nitrate nitrogen and transform it into proteins. The nature of the organism, the amount and nature of energy source, as well as the environmental conditions, influence the amount of nitrate thus assimilated.

The fungi readily utilize nitrate-nitrogen, although often not to such an extent as ammonia nitrogen.<sup>11</sup> For every 30 to 40 units of carbohydrate decomposed, certain fungi assimilate one part of nitrogen. The nitrate is usually first reduced to ammonia before it is assimilated. Only certain groups of bacteria (so-called "nitrate" bacteria) are capable of utilizing this source of nitrogen. The actinomyces assimilate nitrate readily, but usually reduce it first to nitrite; carbon sources favoring growth also favor nitrate reduction; when nitrite is the sole source of nitrogen, particularly in low concentrations (0.01 to 0.05 per cent), it is assimilated very readily as such. The reduction of nitrate by microorganisms is frequently accompanied by an increase in alkalinity of the medium; the reduced anion is usually assimilated and the cation is left behind. The amount of nitrate-nitrogen converted into microbial protoplasm will thus depend upon the nature of the organisms

<sup>10</sup> Vogel, J. Centrbl. Bakt. II, **32**: 169-179. 1912; Bierema, 1909 (p. 426).

<sup>11</sup> Klein, G., Eigner, A. and Müller, H. Ztschr. physiol. Chem., **159**: 201-234. 1926.



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<sup>8</sup> Carapelle, E. Centrbl. Bakt. I, Orig., 47: 545-559. 1908.

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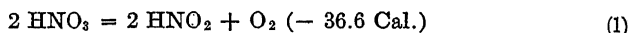
<sup>10</sup> Vogel, J. Centrbl. Bakt. II, 32: 169-179. 1912; Bierema, 1909 (p. 426).

<sup>11</sup> Klein, G., Eigner, A. and Müller, H. Ztschr. physiol. Chem., 159: 201-234. 1926.

active in the process as well as upon the environmental conditions. With citric acid as a source of energy, it was found<sup>12</sup> that a pure culture of a bacterium (*Bact. putidum*) assimilated, under anaerobic conditions, about ten per cent of the nitrate nitrogen in the medium, but, under aerobic conditions, nearly thirty-three per cent of the nitrogen was assimilated, due to the greater utilization of the energy.

*Utilization of nitrates by microorganisms as sources of oxygen.* Certain bacteria are capable of reducing nitrates to nitrites, ammonia, and atmospheric nitrogen or oxides of nitrogen. Goppelsröder<sup>13</sup> was the first to observe that nitrates are reduced in the soil to nitrites; he ascribed this property to the organic matter of the soil. However, Schoenbein in 1868 and Meusel in 1875 recognized the bacterial nature of the process.<sup>14</sup> This idea was developed further by Gayon and Dupetit and others, as shown previously (p. 154).

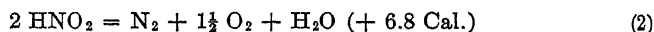
In the absence of free oxygen but in the presence of nitrate, various aerobic bacteria are capable of existing anaerobically. Some organisms bring about complete denitrification; others reduce the nitrate to the nitrite stage only, with a smaller amount of oxygen becoming thereby available.



If we assume that one mole of oxygen can liberate 112 Calories, when carbohydrates are used as a source of energy (with complete oxidation to  $\text{H}_2\text{O}$  and  $\text{CO}_2$ ) a net gain in the above reaction is obtained:

$$112 - 36.6 = 75.4 \text{ Cal.}$$

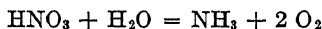
When the nitrate is reduced to atmospheric nitrogen,



The second reaction gives, therefore, a net gain of energy,

$$\frac{1}{2} \times 112 + 6.8 = 174.8 \text{ Cal.}$$

In the reduction of nitrate to ammonia, the following reaction takes place:<sup>15</sup>



<sup>12</sup> Seiser, A. and Walz, L. Arch. Hyg., 95: 189-208. 1925.

<sup>13</sup> Goppelsröder, F. Poggendorf's Annalen, 115: 125. 1862.

<sup>14</sup> Schönbein, C. F. Jour. prakt. Chem., 105: 208-214. 1868; Meusel, E. Compt. Rend. Acad. Sci. 81: 533-534. 1875.

<sup>15</sup> Warburg, O. and Negelein, E. Biochem. Ztschr., 110: 66-115. 1920.

The more complete the reduction of the nitrate, the more oxygen becomes available and, therefore, the greater is the amount of carbohydrate that can be oxidized and the greater is the gain in energy. In the case of many aerobic microorganisms, nitrate can act as the hydrogen-acceptor, whereby it is first reduced to the  $\text{NO}_2$ -ion, and this, through the hypothetical dioxymmonia ( $\text{HON} \cdot \text{HON}$ ), to  $\text{NH}_2 \cdot \text{OH}$  (hydroxylamine) and then to  $\text{NH}_3$ .<sup>16</sup> The first stage of the reduction can be presented as follows:  $\text{HCOOH} + \text{KNO}_3 \rightarrow \text{CO}_2 + \text{HNO}_2 + \text{H}_2\text{O}$ .

In some cases two organisms may participate in the same process, as in the decomposition of cellulose, in the presence of nitrate. One organism reduces the nitrate, in the absence of atmospheric oxygen, and the other decomposes the cellulose: the former organism supplies the oxygen and the latter the energy necessary for the process.<sup>17</sup> Sulfur and thiosulfate may also be used under anaerobic conditions as sources of energy, with nitrate as a source of oxygen. This was first demonstrated by Beijerinck,<sup>18</sup> who assumed that two organisms are concerned in the process, one reducing the nitrate and the other oxidizing the sulfur chemosynthetically. But he later found that only one organism carries out the complete reaction:



About 1 Cal. is produced per gram of nitrate reduced. When a mixture of sulfur (10 per cent), calcium carbonate and  $\text{KNO}_3$  solution (up to 10 per cent) is inoculated with soil, spontaneous and intense gas production takes place, accompanied by slime formation. The gas consists of nitrogen and  $\text{CO}_2$ . In the absence of organic matter and with sulfur as the only source of energy, carbon dioxide of the atmosphere is utilized for the synthesis of the microbial cell substance. The soil was believed to act as a catalyzer which hastens the process, since, on consecutive transfer, the activities of the organism are weakened. Beijerinck<sup>19</sup> believed that the organisms concerned in the process may occur in two physiologically different modifications, which are hereditarily constant when the feeding conditions remain unchanged. One, an autotrophic form which is adapted to inorganic media (sulfur- or thiosulfate-

<sup>16</sup> Blom, J. *Biochem. Ztschr.*, **194**: 392-409. 1928.

<sup>17</sup> Groenewege, 1920 (p. 379).

<sup>18</sup> Beijerinck, 1904 (p. 82); Beijerinck, M. W. and Minkman, D. C. J. *Centrbl. Bakt. II*, **25**: 30-63. 1910.

<sup>19</sup> Beijerinck, M. W. *K. Akad. Wetenschappen. Amsterdam*, **22**: Nos. 9 and 10. 1920.



carbonate-nitrate) and which shows chemosynthesis; the other, an heterotrophic form, which requires organic food. The heterotrophic forms preserve the power of denitrification with organic food. However, these ideas were not based upon any experimental evidence and were largely hypothetical in nature.

Trautwein<sup>20</sup> also found that some soil organisms are capable of oxidizing thiosulfate under aerobic conditions in the absence of nitrate; growth and autotrophic respiration took place anaerobically only when nitrate was present as a source of oxygen.

Nitrate reduction can be brought about readily by a number of soil bacteria, under anaerobic conditions, when carbon complexes are available as sources of energy.<sup>21</sup> Nitrates enable many facultative anaerobes

TABLE 46

*Influence of nitrate upon the decomposition of sucrose (4 per cent) in nutrient bouillon, under anaerobic conditions (Mazé)*

	BACT. LACTIS AEROGENES		PNEUMOBACILLUS OF FRIEDLANDER	
	Nitrate present	Nitrate absent	Nitrate present	Nitrate absent
	cc.	cc.	cc.	cc.
Volume of gas produced.....	628.4	1715.15	548.9	1118.9
CO <sub>2</sub> per 100 of gas.....	57.6	64.7	69.3	62.9
H <sub>2</sub> per 100.....	0	33.4	0	35.0
NO per 100.....	6.7	0	1.2	0
N <sub>2</sub> O per 100.....	2.3	0	0	0
N <sub>2</sub> per 100.....	23.4	—	29.5	—

to develop under anaerobic conditions, using sources of carbon which would otherwise not be available.<sup>22</sup>

According to Mazé,<sup>23</sup> nitrate reduction is caused by the hydrogen produced by anaerobic bacteria; however, not all hydrogen-forming organisms are capable of reducing nitrate, as in the case of butyric acid bacteria. Table 46 illustrates the rôle of nitrate in the decomposition of carbohydrate (4 per cent sucrose) under anaerobic conditions and in a nutrient bouillon; a much greater decomposition of the sugar and an abundant formation of hydrogen in the absence of nitrate points to a

<sup>20</sup> Trautwein, 1924 (p. 85).

<sup>21</sup> Van Iterson, G. Centrbl. Bakt. II, 12: 106-115. 1904.

<sup>22</sup> Ritter, G. Centrbl. Bakt. II, 20: 21-38. 1908.

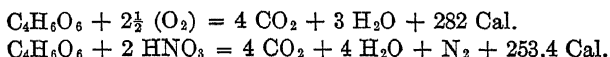
<sup>23</sup> Mazé, 1911 (p. 155).

distinct difference in the mechanism of the decomposition of the substrate.

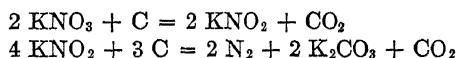
*Reduction of nitrates to gaseous nitrogen and oxides of nitrogen.* The reduction of nitrates to atmospheric nitrogen always goes through the nitrite stage. The following reaction was at first suggested<sup>24</sup> to explain the complete reduction of the nitrate molecule:



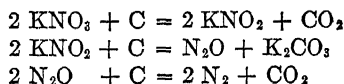
The carbohydrates or organic acids of the media are decomposed with the formation of carbon dioxide and nascent hydrogen;<sup>25</sup> the nitrate is then used by the organism as the hydrogen acceptor, which results in the reduction of the nitrate. When tartaric acid is oxidized by atmospheric oxygen or by reduction of nitrates, nearly equal amounts of energy are liberated, since the reduction of nitrates to atmospheric nitrogen does not consume a large amount of energy.



The theories concerning the nitrate reduction current about 1910 illustrate the reactions involved as follows:



C designates the carbon source. In view of the fact that oxides of nitrogen are always produced in the complete reduction of the nitrate the above reactions had to be modified:<sup>26</sup>



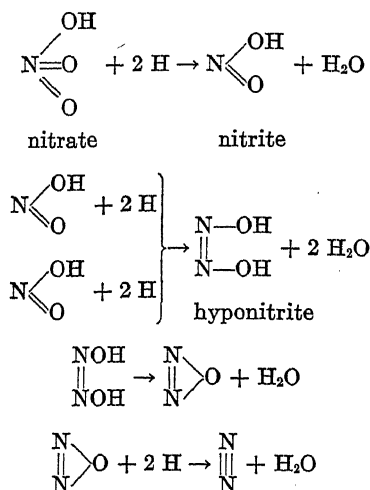
An abundant formation of  $\text{N}_2\text{O}$  takes place at a high nitrate concentration of the medium and at a high temperature. The above reactions are altogether hypothetical. The more recent ideas concerning the

<sup>24</sup> Gayon and Dupetit, 1882 (p. 154); Dehérain, P. P., and Maquenne, L. Compt. Rend. Acad. Sci., 95: 691. 1882.

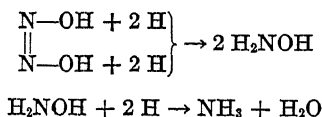
<sup>25</sup> Stoklasa, J. and Vitek, E. Centrbl. Bakt. II, 14: 102-118, 183-205. 1905.

<sup>26</sup> Beijerinck and Minkman, 1910 (p. 479).

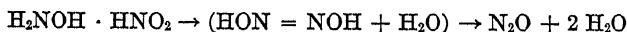
processes involved in the reduction of nitrates can be best presented as follows.<sup>27</sup>



To explain the formation of hydroxylamine and ammonia, in the reduction of nitrates, by the above theories, the following reactions may be suggested:



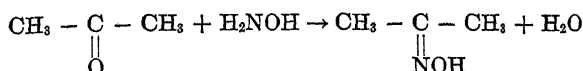
Hydroxylamine is a very labile and highly reactive compound. One cannot expect, therefore, that there will be an accumulation of this compound in the medium. It should be considered as an intermediary product in the reduction of  $\text{NO}_3^-$  ions to  $\text{NH}_4^-$  ions.



Blom suggested various methods for determining the presence of hydroxylamine in culture media even in very small traces. The addition of a

<sup>27</sup> Blom, J. N. J. F. Kongr. Oslo. 1926; *Biochem. Ztschr.*, **194**: 385-391, 392-409. 1928; see also Kluyver and Donker, 1926 (p. 458); Franzen and Löhmann. *Ztschr. physiol. Chem.*, **63**: 102. 1909; Kostytschew and Tswetkova. *Ibid.*, **111**: 198. 1920. Joss, E. J. *Jour. Phys. Chem.*, **30**: 1222-1275. 1926.

small amount of acetone (0.4 cc. per liter of medium) serves the purpose of combining with hydroxylamine, thus obtaining it in larger concentrations than would be found in a free state. Acetoxin is formed as a result of this reaction:



Most of the denitrifying bacteria reduce nitrate to nitrogen gas and  $\text{N}_2\text{O}$ , in varying proportions, *Bac. nitroxus* being particularly active in the process. A 5 to 12 per cent solution of nitrate inoculated with

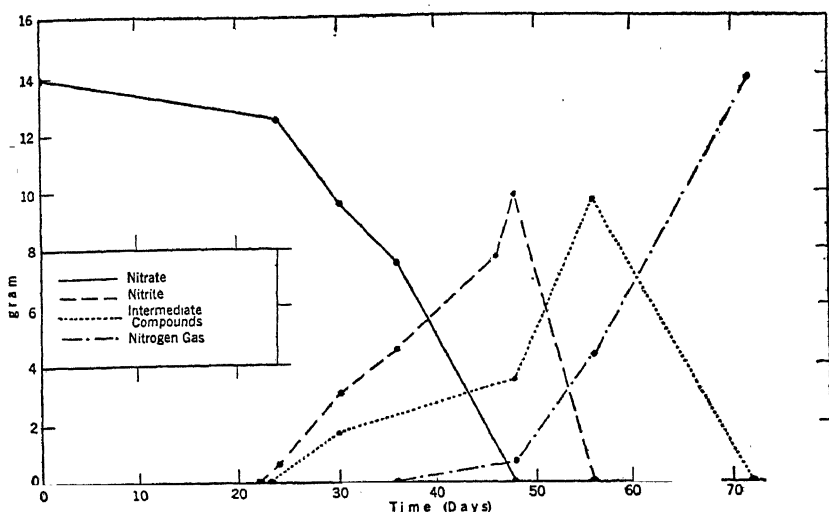


FIG. 34. Reduction of nitrate by microbes (after Korsakowa)

soil gives, at  $20^\circ$  to  $37^\circ$ , a current of gas which is eighty per cent  $\text{N}_2\text{O}$ . Various other denitrifying bacteria, like *Bact. pyocyaneum* and *Bact. stutzeri*, give in solutions of nitrate (particularly  $\text{NH}_4\text{NO}_3$ ) a gas rich in  $\text{N}_2\text{O}$ . Out of one hundred cultures of bacteria tested by Maassen,<sup>28</sup> thirty-one were found capable of reducing nitrate to nitrite; the latter is then reduced to atmospheric nitrogen and NO. This process was rather slow and independent of the oxygen supply. Tacke<sup>29</sup> found that thirty-eight per cent of the gas mixture formed during the process of nitrate reduction by bacteria may consist of  $\text{N}_2\text{O}$ . The formation of

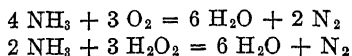
<sup>28</sup> Maassen, 1901 (p. 154).

<sup>29</sup> Tacke, 1888 (p. 156).

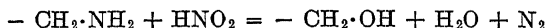
nitric oxide in the reduction of nitrates has also been demonstrated by other investigators.<sup>30</sup>

Fig. 34 brings out clearly the reduction of nitrate, through the nitrite and intermediary compound stages, into nitrogen gas by *Bact. fluorescens liquefaciens*.<sup>31</sup>

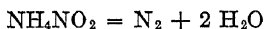
*Formation of nitrogen gas from organic compounds.* To make the study of formation of nitrogen gas in microbiological reactions complete, attention should be called to the possibility of its formation in the decomposition of organic compounds, especially as a result of rapid oxidation of ammonia which is formed from those compounds:



These two processes may play an important part in causing a loss of nitrogen in the decomposition of manure.<sup>32</sup> Nitrogen gas may also be formed by the interaction of nitrites with amino compounds; both of these may be formed in the decomposition of organic matter rich in nitrogen accompanied by the incomplete liberation of ammonia:



The free nitrogen may, of course, be a result of the chemical interaction of the oxides of nitrogen with ammonia:



*Denitrification in the soil.* There is no parallelism between the process of denitrification as it takes place in soil and in solution cultures.<sup>33</sup> In solution and in the presence of the proper organic substances the bacteria may liberate practically all the nitrogen present in the nitrate form as free nitrogen gas, while in moderately moist soil only protein may be formed out of the nitrate. But if the soil is very moist and nitrates are present, denitrifying bacteria behave as in solution and

<sup>30</sup> Lebedeff, A. F. Ber. deut. bot. Gesell., **29**: 327-329. 1911; Suzuki, S. Centrbl. Bakt. II, **31**: 27-49. 1911; Hulme, W. Trans. Chem. Soc., **105**: 623-632. 1914; Acklin, O. Biochem. Ztschr., **164**: 312-370. 1925; see also Lloyd, B. Jour. Roy. Tech. Coll. Glasgow, **2**: 530-550. 1931.

<sup>31</sup> Korsakova, M. P. Bul. Acad. Sci., U. S. S. R. 1927: 1221-1250; 1928: 341-346; 1929: 505-518, 599-605.

<sup>32</sup> Pfeiffer, Th. et al. Landw. Vers. Sta., **48**: 189-245. 1897; Street, J. P. N. J. Agr. Exp. Sta. 14th Ann. Rept: 183-210. 1901; Löhnis, 1910, p. 488 (p. XIV).

<sup>33</sup> Koch, A. and Pettit, H. Centrbl. Bakt. II, **26**: 335-345. 1910.

liberate considerable quantities of free nitrogen gas. The minimum moisture content at which the complete reduction of nitrate may occur depends upon the nature of the soil. Oelsner<sup>34</sup> found that it takes place in soils containing 40 per cent moisture even when no additional carbon has been added. This is especially true of rice fields and of peat soils.<sup>35</sup> The loss of available nitrogen as a result of liming acid peat soil was ascribed<sup>36</sup> to the reduction of nitrate to nitrite and to atmospheric nitrogen. A decrease in aeration leads to an increase in denitrification; cultivation alone could not prevent the loss of nitrogen. The use of disinfectants for the destruction of the denitrifying bacteria in the soil is often recommended.

Denitrification is found to be at an optimum at 25° to 30°. However, it takes place abundantly in the soil even in the coldest seasons of the year when the temperature of the soil at a depth of 0.2 to 0.3 meters is about zero.<sup>37</sup> The optimum reaction for denitrification is at pH 7.0 to 8.2; the process is greatly retarded at pH 5.2 to 5.8 and 8.2 to 9.0.<sup>38</sup>

The denitrifying bacteria (except the forms which obtain their energy from the oxidation of sulfur) require organic matter for their metabolism and are, therefore, favored by an addition of available organic matter. Various mono- and di-basic organic acids (except oxalic) can be utilized as sources of carbon.<sup>39</sup> Glucose is one of the best sources of energy. Fresh straw is utilized to a more limited extent and composted straw even less so, due probably to a slow availability of the more insoluble constituents, since cellulose is not used to any extent as a source of energy by these organisms.<sup>40</sup> A combination of an available source of energy and anaerobic conditions in the soil (either brought about by high moisture content or replacement of oxygen by hydrogen and CO<sub>2</sub>) leads to most active denitrification. An increased nitrate content of a soil favoring conditions of complete denitrification will favor the process further. The addition of large quantities of straw manure and green manure<sup>41</sup> has, therefore, an important influence upon the dis-

<sup>34</sup> Oelsner, A. *Centrbl. Bakt.* II, **48**: 210-221. 1918.

<sup>35</sup> Daikuhara, G. and Imaseki, T. *Bul. Imp. Centr. Agr. Exp. Sta. Japan*, **1**: 7-36. 1907; Ritter, 1912 (p. 659); see also Lemmermann, O. and Wichers, J. L. *Centrbl. Bakt.* II, **41**: 608-625. 1914.

<sup>36</sup> Arnd, 1914-1916 (p. 464).

<sup>37</sup> Barthel, 1909 (p. 469); Krüger and Schneidewind, 1899 (p. 487).

<sup>38</sup> Zacharova, T. M. *Institute of Fertilizers, Moskau*. No. 29. 1925.

<sup>39</sup> Jensen, 1897 (p. 154); Salzmann, P. *Diss. Königsberg*. 1902.

<sup>40</sup> Caron, H. V. *Centrbl. Bakt.* II, **33**: 62-116. 1912; Wright, C. R. *Centrbl. Bakt.* II, **46**: 74-79, 1916; Albrecht, 1922-1926 (p. 613).

<sup>41</sup> Ferguson, M. and Fred, E. B. *Va. Agr. Exp. Sta. Rep.* 1908, 134-149.

appearance of nitrates, both through transformation into protein and reduction to nitrites, ammonia and nitrogen gas. It has been found that even difficultly decomposable organic substances may also have a favorable influence upon denitrification in the soil.<sup>42</sup>

The fact that denitrifying bacteria are favored by an alkaline reaction and are injured by acids suggests the use of substances which would make the soil reaction acid. However, the final reaction should not be acid enough so as to injure the activities of useful bacteria, like the nitrifying organisms. Applications of acid phosphate were found to be useful in the preservation of the manure by neutralizing the ammonia; this brings about a change in reaction and tends, therefore, to lessen denitrification. Of the various nitrates, salts of alkalis and alkali earths are readily denitrified; the reduction of  $\text{Al}(\text{NO}_3)_3$  is doubtful.<sup>43</sup> Iron, manganese, thorium, yttrium and silver nitrates give negative results, due to the toxic action of the cations. Ethyl nitrate is reduced, but not nitro-methane. The same bacteria that reduce nitrates were found capable of reducing potassium chlorate, arsenate and potassium ferricyanide.

*Importance of nitrate reduction in the soil.* The presence of bacteria in the soil capable of reducing nitrates to atmospheric nitrogen and oxides of nitrogen was definitely established in 1882 by Gayon and Dupetit and Dehérain and Maquenne. Lawes, Gilbert and Warington pointed out the same year that considerable quantities of nitrogen may be given off when a soil receives heavy applications of manure and is saturated with water or is improperly aerated. Bréal<sup>44</sup> announced in 1892 that many substances of organic origin, especially straw, can serve as sources of energy which would enable the bacteria to liberate atmospheric nitrogen from nitrates. This is seen from table 47, where 2 grams of straw and sodium nitrate were added to 400 cc. of water. Bréal emphasized the conclusion that denitrification is not of any importance in normal soils, but may become so in humus-rich forest soils. In 1895 Wagner<sup>45</sup> reported that the addition of manure to liquid cultures containing nitrates greatly increased denitrification; this observation led him to the conclusion that the same process takes place in the soil. He

<sup>42</sup> Nolte, O. Centrbl. Bakt. II, **49**: 182-184. 1919.

<sup>43</sup> Ampola, G. and Ulpiani, C. Gazz. chim. ital. Rome, **29**, pt. 1: 49-72. 1899; **34**, pt. 2: 301-315. 1904.

<sup>44</sup> Bréal. Compt. Rend. Acad. Sci., **114**: 681-683. 1892; Ann. Agron., **18**: 181; **22**: 32; Dehérain, 1902 (p. 767).

<sup>45</sup> Wagner, P. Deut. landw. Presse, **22**: 98, 123, 212. 1895; see also 1897 (p. 157).

found confirmation in this in field experiments where organic nitrogen and nitrates were added simultaneously before the crop was planted. Wagner declared, on the basis of these experiments, that denitrification may take place extensively in cultivated soils; the application of manure (cow or horse) to the soil may often be not only unprofitable but even harmful; this was believed to be due to the fact that manure carries microorganisms which destroy the nitrates in the soil, not only added as such, but even those formed by the nitrifying bacteria. Maercker<sup>46</sup> confirmed Wagner's ideas and explained the variable effects of manure by the varying numbers of nitrate-decomposing bacteria that it contains.

These, as well as similar other investigations, created the impression that when nitrates are added to the soil denitrification sets in and may cause an injurious action by causing the transformation of the nitrate

TABLE 47

*Influence of straw upon denitrification in solution*

	MILLIGRAMS
Nitrogen content of straw.....	9.7
Nitrogen content of nitrate added.....	26.0
Total nitrogen added.....	35.7
Nitrogen found at end of experiment.....	27.1
Nitrogen lost into the atmosphere.....	8.6

into gaseous nitrogen. It was soon found that these results were greatly exaggerated.<sup>47</sup> Losses of nitrogen were found possible only when considerable amounts of organic matter are added together with the nitrate, but this is not commonly done.<sup>48</sup> Pfeiffer and Lemmermann<sup>49</sup> demonstrated that very little actual denitrification takes place in the soil as a result of addition of manure. The lack of nitrogen often observed is due to other causes rather than to the actual loss of nitrogen.<sup>50</sup> Ni-

<sup>46</sup> Maercker. Koch's Jahresber., 2: 216. 1898.

<sup>47</sup> Burri, R., Herfeldt, E. and Stutzer, A. Jour. Landw., 42: 329-384. 1894; Dehérain. Ann. Agron., 21: 501. 1895.

<sup>48</sup> Warrington, R. Jour. Roy. Agr. Soc., 8: 577-607. 1897.

<sup>49</sup> Pfeiffer, Th. and Lemmermann, O. Landw. Vers. Sta., 50: 115-142. 1898; Lemmermann and Wichers, 1914 (p. 485).

<sup>50</sup> Krüger, W. and Schneidewind, W. Landw. Jahrb., 28: 217-252. 1899. 29: 747-770. 1900.



trate reduction sets in when the soil is saturated with water. Only in the presence of a great abundance of organic manures is there any fear of loss of nitrate-nitrogen from the soil in a gaseous form.<sup>51</sup> When soils are submerged under water, the nitrates are rapidly reduced. Nitrites may not be formed at all or only in such small amounts as to be insufficient to cause plant injury. Ammonia is formed in some cases. The reaction of the soil is changed, as a result of this reduction, to more alkaline. A similar increase in alkalinity is observed when green manures are applied to flooded soils.<sup>52</sup> In the case of highmoor peat soils, the addition of lime leads to active nitrification; when the nitrates are reduced by denitrifying bacteria, the soils become rapidly depleted in nitrogen.<sup>53</sup>

Great losses of nitrogen may take place in a humid, hot climate;<sup>54</sup> the rate of loss is increased by liming; bare fallows in rainy season were found to be especially wasteful because of the leaching of nitrates in drainage waters. There is little danger from denitrification in normal soils.<sup>55</sup> The partial reduction of nitrates to nitrites and ammonia, which is more extensive and carried out by larger numbers of microorganisms does not involve any actual losses of nitrogen. The nitrates may completely disappear from the medium without any actual loss of nitrogen.<sup>56</sup> The products formed from the nitrates (nitrites and ammonia) can be further acted upon by nitrifying bacteria; the part of the nitrate assimilated by microorganisms is merely stored away in the soil in an organic form.<sup>57</sup>

Attention must be called here to the fact that many of the studies on denitrification were carried out in solution and not in soil. It is known that in very wet soils and in liquids which do not have a ready access to oxygen the bacteria utilize the oxygen from the nitrate molecule for oxidation purposes, while this does not occur in the presence of sufficient oxygen as in well aerated soils. The losses of nitrogen in the

<sup>51</sup> Stoklasa, J. *Centrbl. Bakt.* II, 17: 27-33. 1906; Fischer, H. *Landw. Jahrb.*, 41: 755-822. 1911.

<sup>52</sup> Karlsen, A. *Bergens Mus. Aarbok.* No. 4. 1927.

<sup>53</sup> Arnd, Th. *Landw. Jahrb.*, 47: 371-442. 1914.

<sup>54</sup> Meggitt, A. A. *Mem. Dept. Agr. India*, 7: 31-53. 1923.

<sup>55</sup> Voorhees, E. B. *N. J. Agr. Exp. Sta. Rpts.*, 12: 97-119. 1899; 13: 88-110. 1900; 14: 144-154, 183-211. 1901; 15: 133. 1902; 16: 148. 1903; 17: 191. 1904; *Jour. Amer. Chem. Soc.*, 24: 785-823. 1902.

<sup>56</sup> Lemmermann, O. (*Habilitationsschrift*), Jena. 1900.

<sup>57</sup> Gerlach and Vogel. *Centrbl. Bakt.* II, 7: 609-623. 1901; *Centrbl. Bakt.* II, 13: 706-715. 1904; Löhnis, F. *Ibid.*, 14: 582-604, 713-723. 1905.

manure compost were found<sup>58</sup> to be due largely to the presence of nitrate forming bacteria. When these bacteria are eliminated or conditions are made unfavorable for their action the losses are considerably reduced. The presence of the bacteria resulted in an increase in the loss of nitrogen from 6.28 to 23.75 per cent in the case of cow manure and from 0.73 to 11.66 per cent in the case of horse manure.<sup>59</sup>

It is often observed that the addition of large quantities of undecomposed organic matter to a soil particularly rich in carbohydrates and poor in nitrogen injures crop growth. This is not due to denitrification to which it has often been ascribed, but to the fact that, in the presence of an excess of available organic matter, the fungi, actinomyces, and various heterotrophic bacteria synthesize an extensive protoplasm. For this purpose, they assimilate the nitrates and ammonium compounds present in the soil and thus compete with higher plants.

The conclusion may be reached that the phenomenon of denitrification is of no economic significance in well aerated, not too moist soils, in the presence of moderate amounts of organic matter or nitrate. However, in the case of soils kept under water for some time, as rice soils, the addition of nitrates may even prove injurious due to the formation of toxic nitrite.<sup>60</sup> It may be added here that there is also no distinct parallelism between plant communities, the geological substrate and the presence and activities of denitrifying bacteria.<sup>61</sup>

*Reduction of other oxygen-rich compounds in the soil.* Among the various inorganic, oxygen-rich compounds which can be readily reduced by microorganisms, the following may be mentioned in addition to nitrates and nitrites: sulfates,<sup>62</sup> sulfites, selenates, selenites, tellurates and phosphates. Hydrogen sulfide is formed not only from sulfates but also from elementary sulfur, sulfites and polythionates. The reduction of selenium compounds, including selenious acid<sup>63</sup> and various

<sup>58</sup> Niklewski, 1923 (p. 627); Smirnow, V. G. Zhur. Opit. Agron., 16: 329-386, 1915.

<sup>59</sup> A detailed review of the subject is given by Löhnis, 1910 (p. XIV); see also Russell and Richards. Jour. Agr. Sci., 8: 495-563. 1917.

<sup>60</sup> Nagaoka, M. Bul. Coll. Agr. Tokyo, 6: No. 3. 1904; Kelley, W. P. Hawaii Agr. Exp. Sta. Bul. 24. 1911; Subrahmanyam, V. Ind. Jour. Agr. Sci., 17: 429-448. 1927.

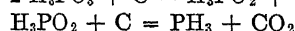
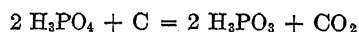
<sup>61</sup> Janssen, G. and Metzger, W. H. Jour. Amer. Soc. Agron., 20: 459-476. 1928.

<sup>62</sup> Saltet, R. H. Centrbl. Bakt. II, 6: 648-695. 1900; van Delden, A. Centrbl. Bakt. II, 11: 81-94, 113-119. 1903; Kochmann, R. Biochem. Ztschr., 112: 255. 1920.

<sup>63</sup> First reported by Japha, A. Diss. Halle. 1842; Chabrié, C. and Lapicoque, L. Compt. Rend. Acad. Sci., 110: 152. 1890; Scheurlen, E. Ztschr. Hyg., 33: 135-136. 1900.

salts as well as tellurium salts, to their elements has been demonstrated for a number of bacteria and fungi. The amount of reduction was found<sup>64</sup> to be proportional to the growth of the organisms. Selenic acid is reduced in two stages, first to selenious acid and then to free selenium.

The reduction of arsenic to arsine is carried out by *Pen. brevicaulis*, an organism found in the soil,<sup>65</sup> as well as by certain other organisms. Various bacteria are also capable of reducing organic<sup>66</sup> and inorganic phosphorus compounds. According to Rudakov,<sup>67</sup> mineral phosphates are reduced, under anaerobic conditions, to phosphites ( $\text{H}_3\text{PO}_3$ ) and hypophosphites ( $\text{H}_3\text{PO}_2$ ) as well as to phosphene. Pure cultures were obtained of a bacterium capable of bringing about the reduction of the phosphate. Different soils varied in their capacity of bringing about this reduction. The addition of  $\text{KNO}_3$  and  $\text{MgSO}_4$  to the medium led to a diminution of the reduction of phosphate, since the activities of the reducing microorganisms were directed towards the more readily reduceable compounds. Carbon compounds were used as sources of energy:



Liebert<sup>68</sup> calculated as a result of thermo-chemical considerations, that the reduction of phosphates, in the presence of mannitol, does not liberate any energy for the activities of the bacteria. This process of reduction of phosphate, even if it takes place cannot, therefore, be considered as one similar to denitrification; Liebert could not demonstrate any reduction products of phosphoric acid. Rudakov's results were, however, confirmed by other investigators<sup>69</sup>

The reduction of various organic compounds in the soil, under anaerobic conditions, is very common, as pointed out previously.

<sup>64</sup> Klett, A. Ztschr. Hyg., **33**: 137. 1900; Levine, V. E. Jour. Bact., **10**: 217-264. 1925.

<sup>65</sup> Gosio, B. Riv. d'igiene, **3**: 201. 1892; Abel, R. and Buttenberg, J. Ztschr. Hyg., **32**: 449. 1899.

<sup>66</sup> Barrenscheen, H. K. and Beckh-Widmenstetter, H. A. Biochem. Ztschr., **140**: 279-283. 1923.

<sup>67</sup> Rudakov, K. I. Viestnik Bact. Agron. Sta., **26**: 171-188. 1926; **28**: 203-232. 1928. Centrbl. Bakt. II, **70**: 202-214. 1927; **79**: 229-245. 1929.

<sup>68</sup> Liebert, F. Centrbl. Bakt. II, **72**: 369-374. 1927.

<sup>69</sup> Horowitz-Wlassowa, L. Centrbl. Bakt. II, **78**: 172-177. 1929.

## CHAPTER XX

### FIXATION OF ATMOSPHERIC NITROGEN BY MICROORGANISMS NON-SYMBIOTIC NITROGEN FIXATION

*Sources of energy.* It has been commonly assumed that the fixation of nitrogen is an endothermic reaction and that the carbohydrates or other available carbon compounds required by the organisms concerned in the process are for the purpose of supplying the required energy. Burk<sup>1</sup> has shown, however, that, when either oxygen or hydrogen gas, or other substances, especially gases whose standard free energies are close to zero, are involved to form compounds like nitrates, ammonia or cyanide, the process of fixation of nitrogen will take place with liberation of energy or free energy. In that case, the carbon requirement of the organism, either in the form of carbohydrate or as organic acids, is largely for the purpose of general microbial metabolism (synthesis of tissues, respiration, etc.), exclusive of the fixation process.

The amount of nitrogen fixed per unit of carbon compound utilized by the bacteria is found to vary, as shown by results obtained by different investigators<sup>2</sup> (fig. 35). Monosaccharides and other simple carbohydrates as well as alcohols and organic acids are the most readily available sources of carbon for nitrogen-fixing bacteria. Polysaccharides, like cellulose, can also serve as valuable sources of energy if they are first partially broken down by cellulose decomposing organisms.<sup>3</sup> These results need still further confirmation; this process is of particular importance, since the bulk of the energy material commonly added to the soil consists of cellulose and pentosans. There are indications in the literature that certain hemicelluloses, including pentosans, can be utilized as direct sources of energy for nitrogen-fixation.<sup>4</sup> The results

<sup>1</sup> Burk, D. Jour. Gen. Physiol., 10: 559-573. 1927.

<sup>2</sup> Gainey, P. L. Ann. Mo. Bot. Gard. 15: 113-168. 1928.

<sup>3</sup> McBeth, I. G. U. S. Dept. Agr. Bur. Pl. Ind. Circ., 131: 25-34. 1913; Pringsheim, 1906-1912 (p. 107); Bottomley, W. B. Proc. Roy. Soc. B., 82: 627-629. 1910; 85: 466. 1912; Koch, A. Centrbl. Bakt. II, 27: 1-7. 1910; 31: 567-570. 1912; Stoklasa, J. Centrbl. Bakt., II, 21: 484-509, 620-632. 1908.

<sup>4</sup> Stránák, Fr. Zeitschr. Zuckerind. Böhmen, 33: 599. 1909 (Centrbl. Bakt. II, 25: 320. 1909); Diehm, R. Proc. III Comm., 2d Intern. Congr. Soil Sci. 1930.

obtained on the relative availability of different energy sources are rather variable, and are subject to numerous influences. By using different periods of incubation, media of different composition and different soils for inoculation (difference in the mixed flora), a different series of results is frequently obtained.

Linhart<sup>5</sup> calculated that only about 1 per cent of the total available energy in mannitol is utilized by *Azotobacter* for the fixation of nitrogen. This was based upon the assumption that *Azotobacter* fixes 10 grams of nitrogen for every gram of mannitol decomposed and that all the

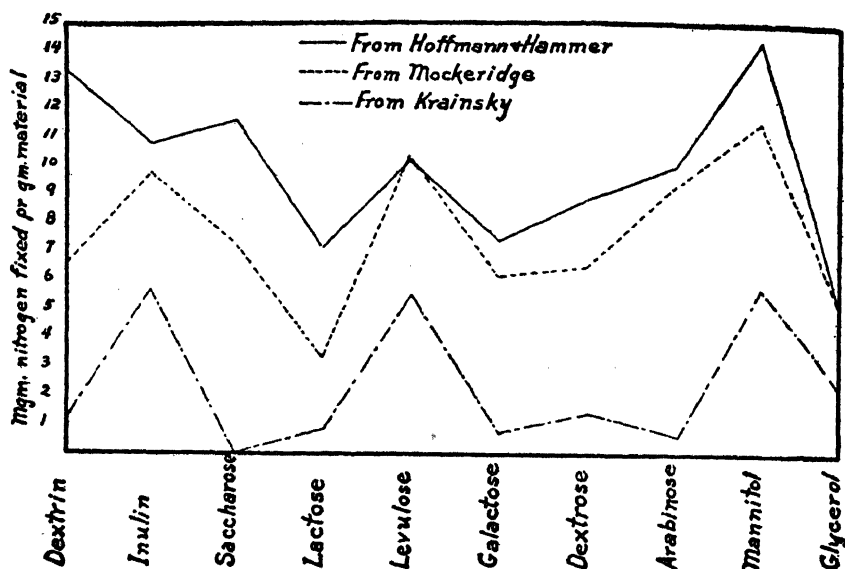


FIG. 35. Showing fixation of nitrogen per gram of organic material, secured by different investigators (from Gainey).

mannitol is converted into  $\text{CO}_2$ . No allowance was made for the formation of intermediary products or the synthesis of microbial protoplasm.

As to the availability of various plant materials as sources of energy for nitrogen fixing organisms, table 48 shows the amounts of nitrogen fixed for 100 grams of carbon by *Az. chroococcum*.<sup>6</sup> A medium containing 1 gram  $\text{K}_2\text{HPO}_4$  and 1 gram  $\text{CaCO}_3$  in 1 liter of tap water was used. Ten grams of organic matter and 250 cc. of medium were placed in liter

<sup>5</sup> Linhart, G. A. Jour. Gen. Physiol., 2: 247-251. 1920.

<sup>6</sup> Dvořák, J. Ztschr. landw. Vers. Sta. Oesterr., 15: 1077-1121. 1912.

flasks; these were sterilized and inoculated with pure cultures of the organism.

The favorable action of the last four substances preceding glucose is due to at least two factors: (1) the abundance of monosaccharides or readily hydrolyzable hemicelluloses in the leguminous plants and fresh materials; (2) the legumes were used fresh and might have exerted, therefore, a more stimulating effect upon the assimilating capacity of the cells, whereas the other substances were all used in a dry condition. Substances especially rich in lignin are not good sources of energy for the activities of the nitrogen fixing organisms.

The amount of nitrogen fixed depends not only upon the nature of the energy source, but also on the presence of available nitrogen, minerals, reaction and other environmental conditions, as well as upon the

TABLE 48  
*Nitrogen fixed by Az. chroococcum for 100 grams of carbon*

	MILLI-GRAMS		MILLI-GRAMS
Pine needles.....	57.3	Plant roots and stubble.....	596.8
Oak leaves.....	126.9	Lupines.....	711.5
Maple leaves.....	89.5	Alfalfa.....	319.5
Wheat straw.....	325.4	Clover.....	1237.9
Corn stover.....	280.3	Glucose.....	1456.5

specific bacteria.<sup>7</sup> Some species utilize more readily one source of energy than another. The amount of nitrogen fixed depends upon the energy value of the particular compound as well as the nature of its decomposition. Lipman<sup>8</sup> recorded an increase in the amount of nitrogen fixed with an increase in molecular weight of fatty acids, in the form of sodium salts, namely, acetic, propionic, butyric; the next member of the homologous series (valerianic acid) presented a poor source of carbon; the sodium salts of succinic and citric acids were not utilized at all. Mockeridge<sup>9</sup> obtained 6.08 mgm. of nitrogen fixed with butyric acid and 1.47 mgm. with formic acid as sources of energy. There was nearly a

<sup>7</sup> Löhnis, F. and Pillai, N. K. Centrbl. Bakt. II, 20: 781-800. 1908; Krainsky, A. Zhur. Opit. Agron., 9: 689. 1908; (Centrbl. Bakt. II, 20: 725-736. 1908); Hoffmann, C. and Hammer, R. W. Wis. Agr. Exp. Sta. Res. Bul. 12. 1910; Centrbl. Bakt. II, 28: 127-139. 1910.

<sup>8</sup> Lipman, 1903 (p. 111).

<sup>9</sup> Mockeridge, F. A. Biochem. Jour., 9: 272-283. 1915; Ann. Bot., 26: 871-887. 1912.

constant ratio between the amount of nitrogen fixed and the heat of combustion of fatty acids; the heat of combustion of butyric acid is 5.96 calories per gram and of formic acid 1.37 calories. Benzene derivatives and most glucosides seem to be unsuitable as sources of energy for *Azotobacter*.<sup>10</sup>

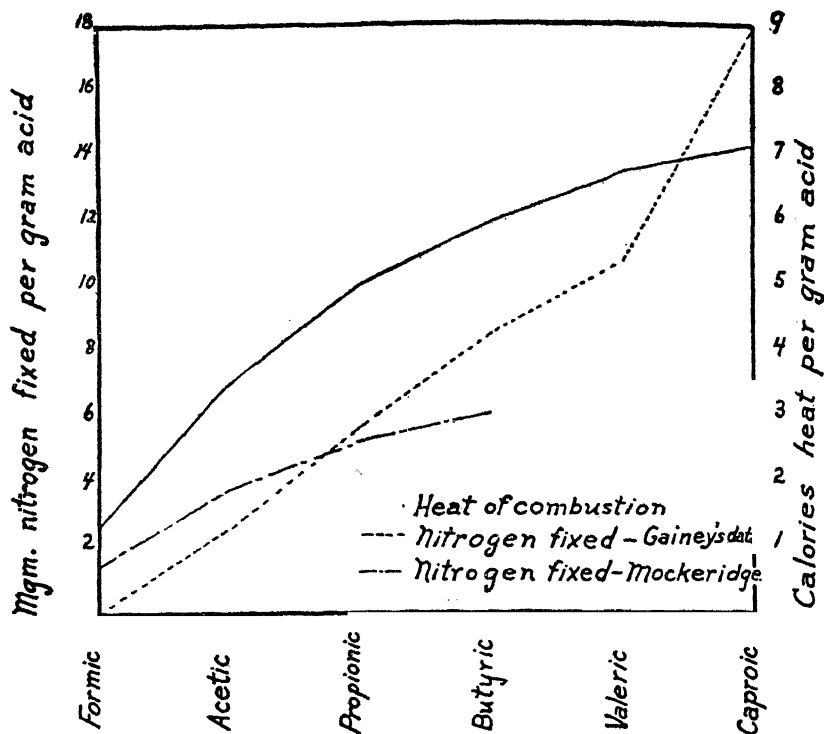


FIG. 36. Comparison between nitrogen fixation and heat of combustion per gram of organic acid, used as a source of energy (from Gainey).

According to Gainey,<sup>11</sup> various strains of *Azotobacter* behave differently with respect to their ability to utilize different fatty acids; some strains can utilize only few acids while others can consume all the acids tested. The iso-acids are not as readily utilized as the normal acids. The relation between the quantity of nitrogen fixed and the heat of combustion of the acid is shown in fig. 36. The amount of nitrogen

<sup>10</sup> Greaves, J. E. *Soil Sci.*, 6: 163-217. 1918.

<sup>11</sup> Gainey, 1928 (p. 491).

fixed should be calculated on the basis of energy utilized and not on the basis of sugar consumed. By comparing two strains of *Clostridia*, one anaerobic and the other aerobic, Pringsheim<sup>12</sup> reported that the difference in the amount of nitrogen fixed per unit of sugar consumed is due to the relative amounts of acids and gases formed. The anaerobic organism produced 45 per cent acid and 55 per cent gas out of the sugar consumed. The aerobic strain produced only 33 per cent acid and 67 per cent gas because of the more complete decomposition of the organic matter; this strain fixed more nitrogen. The ratio between the sugar changed into gas and the nitrogen fixed was nearly the same for both organisms, namely, 23 and 21. The differences in the consumption of sugar by the different organisms cannot serve, therefore, as criteria. *Azotobacter* fixes as much as 3 to 20 mgm. of nitrogen per gram of sugar consumed, while *Cl. pastorianum* fixes a maximum of 2 to 3 mgm. nitrogen for the same amount of sugar. When the actual energy liberated is compared, the latter organism may be found to be more efficient.

*Chemistry of decomposition of carbohydrates.* The anaerobic nitrogen-fixing bacteria decompose carbohydrates and their derivatives with the formation of various acids, chiefly butyric and acetic, and various gases. One liter of medium containing forty grams of glucose inoculated with the anaerobic organism and placed in a nitrogen atmosphere showed a gain of 53.6 mgm. of nitrogen in 20 days. All the sugar disappeared, giving rise to 3.714 grams acetic acid, 14.164 grams *n*-butyric acid,  $\frac{1}{3}$  cc. alcohol, chiefly iso-butyl, and traces of lactic acid. The amount of acids as well as the relation of the gases ( $\text{CO}_2 : \text{H}_2$ ) were found to vary in the different experiments. The gases usually made up 55 to 67 per cent of the sugar decomposed and consisted of 49 per cent carbon dioxide and of 51 per cent hydrogen.<sup>13</sup>

*Azotobacter* decomposes carbohydrates, higher alcohols and organic acids, without the formation of considerable amounts of intermediary products, such as various organic acids;  $\text{CO}_2$  is the only gas formed; the reaction of the medium does not become more acid, but may even become more alkaline because of the utilization of the organic acids present as sources of energy.<sup>14</sup> Some aerobic bacteria may produce ethyl

<sup>12</sup> Pringsheim, H. *Centrbl. Bakt.* II, 20: 248-256. 1908.

<sup>13</sup> Winogradsky, 1893 (p. 101); Omeliansky, W. L. *Monogr.* 5, Russian Acad. Sci. Petrograd. 1923.

<sup>14</sup> Prazmowski, 1912 (p. 110); Omeliansky, W. L., and Ssewerowa, O. P. *Centrbl. Bakt.* II, 29: 643-650. 1911.



alcohol and acetic acid out of the sugar, in the process of nitrogen fixation.<sup>15</sup>

With glucose as a source of energy, *A. chroococcum* was reported<sup>16</sup> to liberate 70 per cent of the C as CO<sub>2</sub>; 12 per cent was assimilated in the bacterial cells, and 18 per cent was left among the various decomposition products other than CO<sub>2</sub>. These were made up of ethyl alcohol, aldehyde, formic, acetic, lactic and tartaric acids and others. The bacterial cells of *Azotobacter* contained 30 per cent protein, a considerable amount of fat and phosphatides.

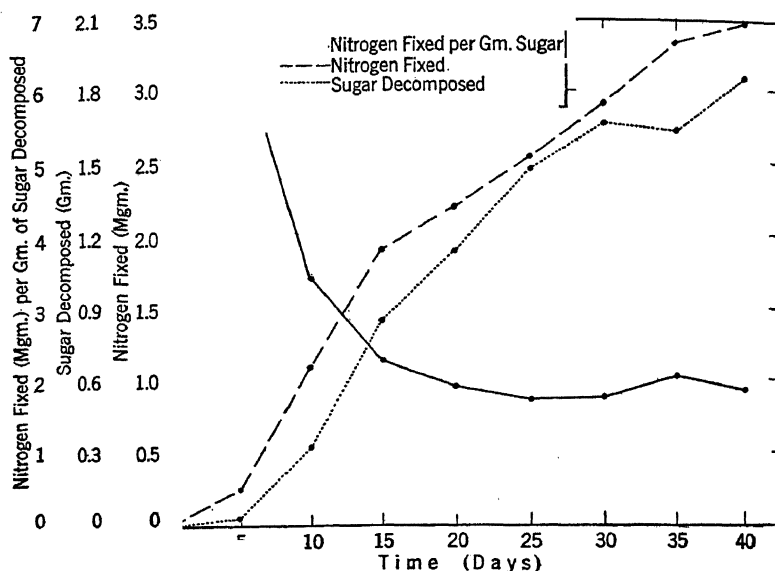


FIG. 37. Relationship between carbohydrate decomposed and nitrogen fixed (after Omeliansky).

The maximum formation of carbon dioxide by *Azotobacter* takes place within the fourth to tenth day of growth. Active CO<sub>2</sub> production may last for 21 to 24 days, and is then followed by a decrease, after 32 to 39 days (fig. 37).<sup>17</sup> In old cultures, the most active CO<sub>2</sub> evolution may be observed even later (on the thirty-sixth day of growth). When

<sup>15</sup> Truffaut, G. and Bezssonoff, N. *La Science du sol.*, 2: 3-16. 1923.

<sup>16</sup> Ranganathan, S., and Norris, R. V. *Jour. Ind. Inst. Sci.*, 10A: 77-96. 1927.

<sup>17</sup> Krainsky, A. B. *Zhur. Obit. Agron.*, 9: 689, 749. 1908; *Univ. Izv. Kiev*, 52: 1-58, 59-131, 133-182. 1912.

aeration is increased, the maximum evolution of  $\text{CO}_2$  takes place at a much earlier period, namely, between the sixth and ninth days.

The differences in the products formed by the various nitrogen fixing bacteria from the carbohydrate used as a source of energy account to a large extent for the difference in the amounts of nitrogen fixed. One gram of glucose liberates 3.7 Calories when oxidized completely to  $\text{CO}_2$  and water; it liberates only 0.08 Calorie when changed into butyric acid and 0.19 Calorie when changed to acetic acid under anaerobic conditions. The energy made available to *Azotobacter*, from the same amount of sugar decomposed, may be forty-six times as much as to *Cl. pastorianum*. The activities of the organisms which fix atmospheric nitrogen result in the consumption of a large amount of energy; the amount of nitrogen fixed is almost proportional to the amount of energy liberated. The organism that is able to liberate a larger amount of energy from the same amount of substrate is also able to fix a larger quantity of nitrogen, even if it is not more efficient.<sup>18</sup>

*Respiration and nitrogen-fixation.* The utilization of energy enables the organism to obtain the nitrogen necessary for the synthesis of its protoplasm from the atmosphere. The amount of nitrogen fixed and the efficiency of the process depend on a series of physical, chemical, and biological factors including temperature, composition and concentration of medium, aeration, age and development of culture, and racial peculiarities.<sup>19</sup> The nature and concentration of the energy source and presence or absence of combined nitrogen are of special importance. The actual quantity of cell substance formed per unit of sugar consumed was found to vary with the stage of growth of the nitrogen-fixing organism. Bonazzi<sup>20</sup> differentiated between the "ferment power" or first stage in the growth of the organism, when nitrogen assimilation is at a maximum, and the second or "maintenance" phase. During this maintenance stage the carbohydrate complexes are actually reworked, partially burned to liberate energy, partially utilized in the building of cellular substance, and partially secreted into the medium in the form of soluble by-products. During the early periods of growth, a unit of cellular substance could utilize in a unit of time 5.45 units of sugar; after an incubation period of 30 days, a unit of cellular substance utilized in a unit of time only 0.28 unit of sugar (fig. 38). Not the sugar itself, but the products of its decomposition form the true sources of energy for *Azotobacter*.

<sup>18</sup> Bonazzi, A. Proc. IV Intern. Soil Sci. Conf. Rome, IIIB. 1924.

<sup>19</sup> Omeliansky, W. L. Arch. Sci. Biol. Petrograd, 18: No. 4. 1914.

<sup>20</sup> Bonazzi, A. Jour. Bact., 6: 331-369. 1921.

The fixation process is usually most efficient in the earlier stages of growth coinciding with the period of greatest cell multiplication and

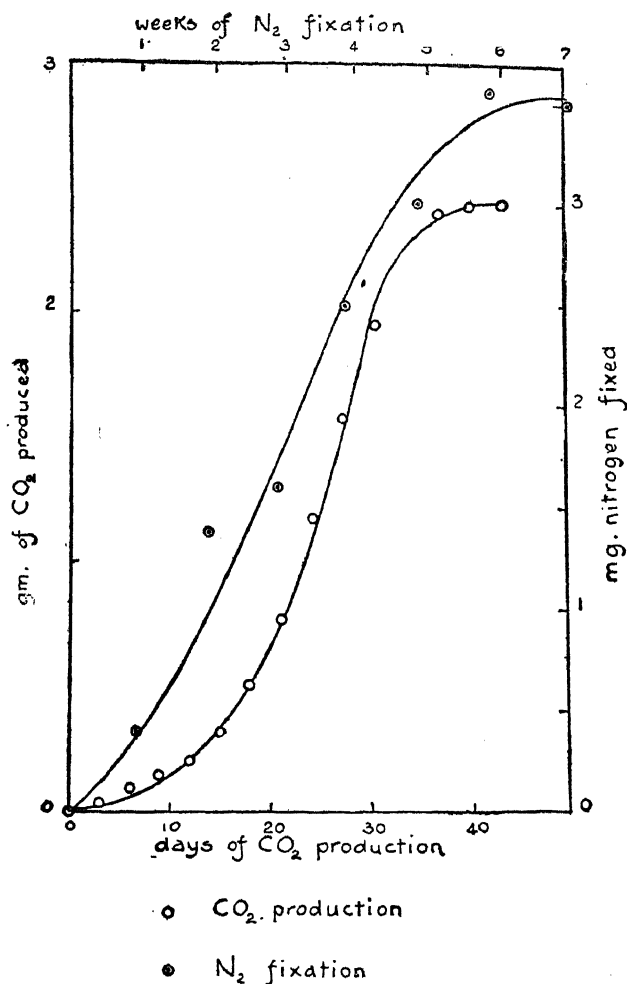


FIG. 38. Rate of nitrogen fixation and energy consumption (after Krainsky and Bonazzi).

sugar utilization. It was found,<sup>21</sup> for example, that 70 to 80 mgm. of nitrogen are fixed by *Azotobacter* per gram of glucose decomposed on

<sup>21</sup> Koch, A. and Seydel, S. *Centrbl. Bakt.* II, 31: 570-577. 1912.

the second and third day of growth, and only 5 to 8 mgm. on the eighth day. Although the total amount of nitrogen fixed during the first five days is small, the process is most economical. In the following 3 to 5 days' periods, the process becomes less and less economical. In the latter stages a larger part of the energy is converted largely into heat. A definite correlation is thus found between the processes of assimilation and dissimilation taking place in the cell and bringing about its development. The rate of growth is more rapid at the early periods and, since nitrogen fixation is a function of the growth of the organism, it will be most active in the early periods; later, however, a great deal of the energy is spent for sustenance of the cells without further growth.<sup>22</sup>

Respiration of *Azotobacter* is influenced by the presence of sugar and is 10–15 times as great in a sugar solution than in a sugar-free solution. Respiration drops per unit of dry weight with increasing age of culture. An increase in the number of bacteria is accompanied by an increase in the dry weight and in nitrogen fixation. Maximum respiration takes place with 15–20 per cent O<sub>2</sub> in the atmosphere. In an atmosphere of pure oxygen, respiration is  $\frac{1}{3}$  to  $\frac{1}{2}$  of that in air. The oxygen concentration has a marked influence upon the ratio of  $\frac{\text{moles N}_2 \text{ fixed}^{23}}{\text{moles O}_2 \text{ used up}}$ .

On increasing the concentration of the energy source in the medium, there is an increase in the amount of nitrogen fixed, but the process is less economical, i.e., less nitrogen is fixed per unit of carbon utilized. When the mannitol contents of the medium were 0.5, 1 and 5 per cent, the corresponding amounts of nitrogen fixed were 11.4, 8.2 and 7.45 mgm. per gram of mannitol oxidized.<sup>24</sup> The following quantities of nitrogen were reported<sup>25</sup> fixed per gram of mannitol in different concentrations of the latter:

Mannitol, per cent.....	0.1	0.2	0.5	1.0	1.5
Nitrogen fixed per gram of mannitol,					
mgm.....	10.5	8.3	6.4	4.68	3.22

The more dilute the concentration of the energy source, the greater is the nitrogen-fixing efficiency of the organisms. This probably accounts for the very efficient energy utilization by the nitrogen-fixing bacteria in the surface layers of the soil. The amount of nitrogen fixed also

<sup>22</sup> Hunter, O. W. Jour. Agr. Res., **23**: 665–677. 1923.

<sup>23</sup> Meyerhof, O. and Burk, D. Ztschr. physik. Chem. A, **139**: 117–142. 1928.

<sup>24</sup> Hoffmann and Hammer, 1910 (p. 493).

<sup>25</sup> Lipman, 1903<sub>4</sub>(p. 111).

depends on the presence of minerals, especially phosphates, of certain stimulating substances and upon the nature of the medium. Nearly ten times as much nitrogen was fixed per unit of sugar consumed in sand as in solution.<sup>26</sup> *Cl. pastorianum* fixed 4.56 mgm. nitrogen per gram of sugar consumed in a 0.5 per cent glucose solution and 1.93 mgm. in a 3.0 per cent sugar solution.<sup>27</sup>

These considerations account for the great variability in the amounts of nitrogen fixed (from 1.2 to 17 mgm.) per gram of sugar consumed by crude and pure cultures of bacteria, as determined by various investigators.<sup>28</sup>

The fixation of 10 mgm. of nitrogen for one gram of energy source consumed is considered<sup>29</sup> as a very efficient process. Thus for every kilogram of nitrogen fixed, the soil loses about 100 kgm. of energy material. If 1 kgm. of material is equivalent to 4000 Calories, 1 kgm. of nitrogen fixed requires an energy consumption of 464 kilowatt hours, greatly in excess of that used in chemical processes (about 46 to 48 kilowatt hours). Such a large expenditure of energy is due to the fact that not all the energy is used by the organism for nitrogen fixation (if at all), but a part of the energy is utilized for metabolic processes and cell activities, including the synthesis of fats, phosphatides, carbohydrates and proteins of a high molecular weight.<sup>30</sup> We must also consider the fact that not all the energy is made available to the nitrogen-fixing bacteria, since the nutrient is not completely broken down to CO<sub>2</sub> and water, but a part is left in the form of intermediate compounds, especially in the case of the anaerobic *Clostridium*. The liberation of hydrogen which is required by the organism for the reduction of the nitrogen also involves a loss in energy.<sup>31</sup>

*Protein synthesis by Azotobacter.* *Azotobacter* was reported<sup>32</sup> to contain 10.45 per cent total nitrogen, of which 6.39 per cent was non-basic N, 2.76 per cent basic N, and 0.98 per cent ammonia N. In young cultures the nitrogen was believed to be present largely in a soluble form which is not precipitated by phosphotungstic acid; as the culture

<sup>26</sup> Krainsky, A. V. Centrbl. Bakt. II, 26: 231-235. 1910.

<sup>27</sup> Omeliansky, 1923 (p. 495); Pringsheim, 1908 (p. 107); Truffaut, G. and Bezssonoff, N. Compt. Rend. Acad. Sci., 175: 868-870. 1921; 177: 649-652. 1923.

<sup>28</sup> Omeliansky, W. L. Arch. Sci. Biol. Petrograd, 19: 162-208. 1915.

<sup>29</sup> Remy, Th. Centrbl. Bakt. II, 22: 561-651. 1909.

<sup>30</sup> Christiansen-Weniger, F. Centrbl. Bakt. II, 58: 41-66. 1923.

<sup>31</sup> Rippel, 1928 (p. 502).

<sup>32</sup> Lipman, 1904-1905 (p. 109); Stoklasa, 1908 (p. 491).

grows older, the material becomes more insoluble. The ash content was reported as 8.6 per cent, 58 to 62.35 per cent of which was phosphoric acid; the nitrogen and the phosphorus are present in the cells chiefly in the form of nucleo-proteins and lecithin, increasing in concentration with the age of the cell.

When the nitrogen-fixing organism is grown on solid media, such as an agar surface, and all the growth collected and dried, a much smaller relative amount of nitrogen is found, amounting to not more than 2 to 3 per cent of the dry weight of the cells.<sup>33</sup> The protein content of *Azotobacter* cells grown on solid media is considerably lower than when grown in liquid media. The difference is due to the fact that a large part of the membranous material and the slime surrounding the cells consist of carbohydrates, free from proteins.<sup>34</sup> This material is filtered out in the case of liquid media; this tends to increase greatly the amount of protoplasm in the residual material.

The phosphorus content was also found to vary from 4.93 per cent  $P_2O_5$  to 2.51 per cent of the dry material. *Azotobacter* grown on agar media contains 0.57 per cent phosphorus and 1.43 per cent potassium.<sup>33</sup> The following analysis of dry *Azotobacter* cells has been reported:<sup>35</sup> moisture 6.63 per cent, ash 4.16 per cent, proteins 12.93 per cent, nitrogen-free material 76.28 per cent. The nitrogen-free materials consist of polysaccharides which show 33 per cent sugar, on hydrolysis with 1.25 per cent  $H_2SO_4$  and 10 per cent  $HCl$ . Beijerinck and Van Delden believed this material to be of the nature of pectin;<sup>36</sup> others reported an abundance of pentosans.<sup>37</sup> The reserve substances of the cell consist of fats and volutin; the slime, a complex carbohydrate, yields a dextro-rotatory fermentable sugar on inversion.<sup>34</sup>

On hydrolysis, the proteins of *Azotobacter* were found to give the following nitrogen groups:

	<i>per cent</i>
Ammonia nitrogen.....	9.9
Melanin nitrogen.....	3.7
Total diamino nitrogen.....	26.4
Total monoamino nitrogen.....	60.0

<sup>33</sup> Hoffmann and Hammer, p. (493). Hunter, O. W. Jour. Agr. Res., **24**: 263-274. 1923.

<sup>34</sup> Stapp, C. Centrbl. Bakt. II, **61**: 276-292. 1924.

<sup>35</sup> Omeliansky, W. L. and Sieber, H. O. Ztschr. physiol. Chem., **88**: 445-459. 1913.

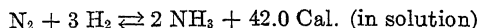
<sup>36</sup> Beijerinck and van Delden, 1902 (p. 101).

<sup>37</sup> Hoffmann, C. Centrbl. Bakt. II, **36**: 474-476. 1913.

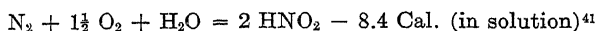
The diämino nitrogen consisted of 14.5 per cent lysine, 10.4 per cent arginine, and 1.6 per cent histidine. The excretion of soluble nitrogen compounds by the nitrogen-fixing organisms is of importance. *Az. chroococcum* was found<sup>38</sup> to autolyze after death and liberate soluble nitrogen compounds. *Az. agile* and *Az. vinelandii*, however, readily excrete such compounds during active growth.

A part of the nitrogen synthesized by *Azotobacter* is readily utilized by higher plants.<sup>39</sup> The proteins of the *Azotobacter* cells are not completely broken down by the soil bacteria and are not readily acted upon by proteolytic enzymes.<sup>40</sup>

*Chemistry of process of non-symbiotic nitrogen-fixation.* Nitrogen shows a strong affinity for the electropositive elements and only a slight affinity for the electronegative elements. The combination of nitrogen and hydrogen is exothermic so that



With increasing temperatures, the ammonia breaks up more and more into its constituents. The combination of nitrogen with oxygen is endothermic:



The oxidation of nitrogen, therefore, requires large quantities of energy and must be carried out at a high temperature so as to obtain the necessary reaction velocity and a favorable equilibrium between  $\text{N}_2 + \text{O}_2 \rightleftharpoons 2\text{NO}$ , which amounts to 5 per cent at 3200° and 10 per cent at 4200°. With an increase in temperature the molecular nitrogen changes to atomic ( $\text{N}_2 \rightleftharpoons 2\text{N}$ ) and brings about an increase of contact between the reacting molecules. In the presence of catalyzers, the reactions are brought about at lower temperatures. The bacteria depend primarily on catalyzers, such as molybdenum.<sup>41a</sup>

Blom<sup>41b</sup> proposed the following reactions to explain the mechanism

<sup>38</sup> Molér, T. *Botan. Notiser*, 4: 163-178. 1915 (*Centrbl. Bakt.* II, 47: 635. 1917).

<sup>39</sup> Kayser, E. *Compt. Rend. Acad. Sci.*, 172: 1539-1541. 1921.

<sup>40</sup> Bonazzi, 1924 (p. 497); Barthel, Ch. *Proc. Second Intern. Congr. Soil Sci.* 1930.

<sup>41</sup> Kostytshew, S. and Schwezowa, O. *Planta*, 2: 527. 1926; according to Rippel, however, even the oxidation of nitrogen is an exothermic reaction:  $\text{N}_2 + 3 \text{O}_2 + \text{H}_2 = 2 \text{HNO}_3 + 97.6 \text{ Cal. (in solution)}$ . Rippel, A. and Poschenrieder, H. *Jour. Landw.*, 76: 101-112. 1928.

<sup>41a</sup> Bortels, H. *Arch. Mikrob.*, 1: 333-342. 1930; Burk, D. and Lineweaver, H. *Ibid.*, 2: 155-186. 1931.

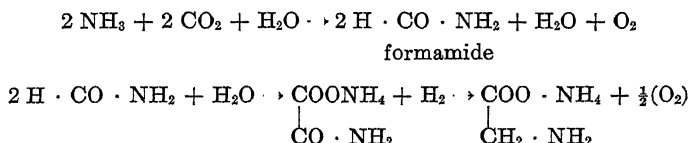
<sup>41b</sup> Blom, J. *Centrbl. Bakt.* II, 84: 60-86. 1931.

of nitrogen fixation by bacteria, through the hydroxylamine stage and with iron as a catalyst:

1.  $N \equiv N$  (atmospheric)  $\rightleftharpoons N \equiv N$  (solution).
2.  $2 (\text{Cat. Fe}^{++}) + N \equiv N \rightleftharpoons (\text{Cat. Fe}^{++})_2 \cdot N \equiv N$ .
3.  $(\text{Cat. Fe}^{++})_2 \cdot N \equiv N + 2 \text{H}_2\text{O} \rightleftharpoons (\text{Cat. Fe}^{++})_2 \cdot \text{HONH} - \text{HNOH}$ .
4.  $(\text{Cat. Fe}^{++})_2 \cdot \text{HONH} - \text{HNOH} + 2 \text{H}^+ \rightleftharpoons 2 (\text{Cat. Fe}^{+++}) + 2 \text{HO NH}_2$ .
5.  $(\text{Cat. Fe}^{+++}) + \text{H} \rightleftharpoons (\text{Cat. Fe}^{++}) + \text{H}^+$ .

Winogradsky<sup>42</sup> believed that, in the case of the anaerobic *Clostridium*, the bacterial plasma produces ammonia out of the nitrogen gas and nascent hydrogen with which it comes into contact. The hydrogen is formed in the butyric acid fermentation of the organism. Winogradsky later demonstrated that *Azotobacter* growing on silica gel media, with sodium salts of organic acids as sources of energy, synthesizes ammonia as the first product of the reaction. When pH approaches 9.0, red litmus paper suspended in the dish above the culture turns blue.<sup>43</sup> Wieland<sup>44</sup> considered that the action of the hydrogen acceptors formed in the cells of nitrogen-fixing bacteria does not depend upon oxygen for hydration, but upon the molecular nitrogen with which it forms ammonia, perhaps through the hydrazine stage in a manner similar to the Haber synthesis.

The ammonia is assimilated by the organism and is converted into protein. The actual assimilation of the nitrogen may take place according to the following reactions:<sup>45</sup>



The fixation of nitrogen by an oxidation process, similar to nitrogen-fixation in moist air through the oxidation of organic matter, has also been suggested.<sup>46</sup> The nitrogen is believed to be first oxidized to  $\text{N}_2\text{O}_3$

<sup>42</sup> Winogradsky, 1893-1895 (p. 101); *Compt. Rend. Acad. Sci.*, **190**: 661-664. 1930.

<sup>43</sup> See also Koztytschew, S. and Ryskaltshouk, A. *Compt. Rend. Acad. Sci.*, **180**: 2070-2072. 1925; *Ztschr. physiol. Chem.*, **154**: 1-17. 1926; **198**: 105-114. 1931.

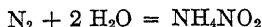
<sup>44</sup> Wieland, H. *Ber. deut. chem. Gesell.*, **55**: 3639-3648. 1922.

<sup>45</sup> Loeb, W. *Ber. deut. chem. Gesell.*, **46**: 684-697. 1913.

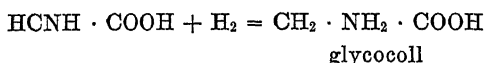
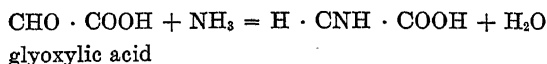
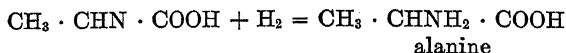
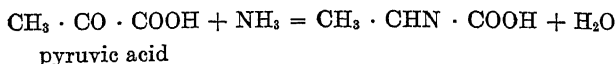
<sup>46</sup> Gautier, A. and Drouin, R. *Compt. Rend. Acad. Sci.*, **106**: 754-757, 863, 944, 1174, 1232, 1605. 1888; **113**: 820-825. 1891; also Berthelot, 1899 (p. 100); Bonnema, A. *Chem. Ztg.*, **27**: 148. 1903. *Centrbl. Bakt. II*, **10**: 598-602. 1903.



with iron hydrate as a catalyst; the nitrous acid may then be assimilated by the organism. Czapek<sup>47</sup> believed that ammonium nitrite may first be synthesized. However, neither nitrate nor nitrite could be found in the cells of *Azotobacter*.<sup>48</sup> Loew and Azo<sup>49</sup> considered the reaction of nitrogen-fixation by *Azotobacter* to take place as follows:



The ammonium nitrite is reduced to ammonia which interacts with the decomposition products of carbohydrates to give amino acids.



By the process of condensation, the amino acids are built up into proteins. Beijerinck and van Delden<sup>50</sup> originally thought that the nitrogen is first fixed in the form of an inorganic soluble compound which goes into solution. No soluble inorganic nitrogen compounds could be demonstrated in pure cultures, but in crude cultures the proteins may again be hydrolyzed into soluble forms of nitrogen. Soluble organic nitrogen compounds were found,<sup>51</sup> however, not only in the filtrate of a phosphotungstic acid precipitate of a dead *Azotobacter* culture, but also after the culture was filtered through a Chamberland filter. It was suggested<sup>52</sup> that *Azotobacter* forms, in its early stage of development, a complex consisting of sugar and nitrate which is similar to the sugar-phosphoric acid complex which takes an active part in alcoholic fermentation. That complex is later used partly for energy and partly

<sup>47</sup> Czapek, F. *Ergeb. Physiol.* I, 2: 638-672. 1903; Heinze, B. *Centrbl. Bakt.* II, 12: 364. 1904.

<sup>48</sup> Kellerman, K. F. and Smith, N. R. *Centrbl. Bakt.* II, 40: 479. 1914.

<sup>49</sup> Loew and Aso. *Bull. Coll. Agr., Tokyo*, 7: No. 5 (*Centrbl. Bakt.* II, 22: 452. 1909); Lipman, 1904-5 (p. 109).

<sup>50</sup> Beijerinck and van Delden, 1902 (p. 101).

<sup>51</sup> Lipman, 1903 (p. 111).

<sup>52</sup> Bonazzi, 1921 (p. 497).

for synthesis of protoplasm. It was actually demonstrated<sup>53</sup> that a large percentage of the total nitrogen fixed in the first few days of growth of *Azotobacter* consists of amino acid nitrogen. This proves that the elementary nitrogen goes through the simple organic forms before it is changed into protein. It also tends to prove that nitrogen is fixed by combination with hydrogen and not with oxygen, thus insuring much greater economy of energy.

*Influence of available nitrogen compounds upon nitrogen fixation.* The nitrogen fixing bacteria do not depend entirely upon atmospheric nitrogen for their need, but are capable of utilizing combined nitrogen present in the medium. This is a direct result of the difference in growth and nitrogen-fixation pointed out above. Nitrates are readily utilized by *Azotobacter* as sources of nitrogen; accordingly the presence of nitrates in the medium inhibits the fixation of atmospheric nitrogen. Ammonium sulfate and peptone are also available nitrogen compounds. One-half milligram of nitrogen in an available form is sufficient to inhibit completely the fixation process.<sup>54</sup>

The injurious action of the nitrate upon the fixation of nitrogen was considered to be due either to (a) a direct toxic action of the salt upon the growth of the organism, (b) stimulation by nitrate of other organisms in a mixed culture which are antagonistic to *Azotobacter*, and (c) competition by such organisms with *Azotobacter* for the energy supply.

The influence of nitrate may not be the same in soil as in solution culture. By taking the number of bacteria developing and the nitrogen fixed in the nitrate-free cultures as 100, it was found<sup>55</sup> that the addition of 50 mgm. of  $\text{NaNO}_3$  brought about an increase in the numbers from 100 to 3150, while the nitrogen fixed was increased 342 per cent in sterilized soil and 500 per cent in unsterilized soil. The presence of nitrate stimulated greatly the multiplication of the *Azotobacter* organism while it reduced its physiological efficiency. According to Bonazzi,<sup>56</sup> *Az. chroococcum* may fix nitrogen in the absence of such fixed nitrogen and act as a "denitrifying" organism in the presence of nitrates. The nitrogen of humus does not seem to exert, except in high concentrations,

<sup>53</sup> Waynick, D. D. and Woodhouse. Cal Agr. Exp. Sta. Ann. Rpt. 1918-19, p. 62-63; Halversen, W. V. Iowa State Coll. Jour. Sci., 1: 395-410. 1927.

<sup>54</sup> Lipman, 1903 (p. 111); Pringsheim, H. Centrbl. Bakt. II, 40: 21-24. 1914; Hanzawa, J. Centrbl. Bakt. II, 41: 573-576. 1913; Krainsky, 1908-1912 (p. 496); Prazmowski, A. Bull. Intern. Acad. Sci. Cracovie, Math. Nat. Cl. Sc. B, No. 7: 855-950. 1912; Burk, D. and Lineweaver, H. Jour. Bact., 19: 389-414. 1930.

<sup>55</sup> Hills, T. L. Jour. Agr. Res., 12: 183-230. 1918.

<sup>56</sup> Bonazzi, 1921 (p. 497).

any injurious influence upon nitrogen-fixation. This is probably due to the fact that its availability is only limited. As a matter of fact, it is even claimed<sup>57</sup> that the soil organic compounds can be used as sources of energy by nitrogen-fixing bacteria. These results need confirmation and elucidation.

*Influence of salts upon nitrogen fixation.* In addition to an energy source, the presence of certain minerals in minimum concentrations is important for the activities of the nitrogen-fixing bacteria. *Azotobacter* prefers soils containing calcium salts, and races of the organism isolated from those soils were more active than those isolated from unlimed soils. Calcium is looked upon as serving a double purpose: (1) directly for metabolism (in the form of soluble phosphates, as the oxide, carbonate, and in the form of salts of organic and inorganic acids) and (2) for the purpose of neutralizing the acids formed in the metabolism (in the form of oxide, hydroxide or carbonate). Certain calcium salts, especially tricalcium phosphate and the chloride, cannot be utilized.<sup>58</sup> Magnesium salts cannot take the place of calcium.<sup>59</sup> The primary importance of calcium is due, however, not to its direct nutrient quality but chiefly to its buffering properties, and it may serve to some extent as a catalytic agent.<sup>60</sup> Magnesium carbonate is very favorable for the development of *Azotobacter* in solution; its presence discourages also the development of foreign organisms, probably due to their suppression by the magnesium.<sup>61</sup>

Potassium salts favor the development of *Azotobacter*, the minimum quantity of this element corresponding to that of calcium. A minimum of 0.38 mgm. K, 0.36 mgm. Ca and 0.35 mgm. Mg was required by *Azotobacter* for every gram of sugar decomposed. In higher concentrations, potassium salts, as a rule, become more toxic than sodium salts. The latter do not seem to be indispensable for the growth of *Azotobacter*, although the addition of three per cent of NaCl does not injure its development. There are claims in the literature,<sup>62</sup> however, that alkalis, and particularly alkali carbonates, are injurious to nitrogen-fixation; the action of NaCl becomes apparent only when 0.5 to 0.6 part are present in 100 grams of dry soil.  $\text{Na}_2\text{SO}_4$  becomes injurious at 0.25

<sup>57</sup> Lipman, C. B. and Teakle, L. J. H. *Soil Sci.*, **19**: 99-103. 1925.

<sup>58</sup> Christensen, H. R. *Centrbl. Bakt.* II, **17**: 109-119. 1907; **19**: 735-6. 1907.

<sup>59</sup> Fischer, H. *Centrbl. Bakt.* II, **14**: 33-34. 1905; **15**: 235-236. 1906.

<sup>60</sup> Vogel, J. *Centrbl. Bakt.* II, **32**: 411-442. 1912; Krzemieniewski, H. *Bul. Intern. Acad. Sci. Cracovie, Cl. Sci. Math. Nat.* No. 5: 445-448. 1908.

<sup>61</sup> Ashby, 1907 (p. 109).

<sup>62</sup> Lipman, C. B. and Sharp, L. T. *Centrbl. Bakt.* II, **35**: 647-655. 1912.

per cent concentration, while 0.4 to 0.5 per cent of  $\text{Na}_2\text{CO}_3$  inhibits growth of the nitrogen-fixing organisms completely.

Phosphorus compounds greatly accelerate the activities of *Azotobacter*, since they play an important rôle in its metabolism,<sup>63</sup> large quantities of the mineral being required for the synthesis of the cells. The organism utilizes particularly well those soluble phosphates which do not tend to make the soil reaction acid, as in the case of the di- and tri-sodium and potassium phosphates and di-calcium phosphates. The presence of mono-basic-phosphates which serve as buffers and tend to make the reaction of the medium more acid than the minimum for the growth of *Azotobacter* is not favorable. The difficultly soluble tri-basic calcium, iron and aluminum salts are available only according to the degree of their solubility. In the presence of an excess of available energy a definite relation was found to exist between the growth of *Azotobacter* and the phosphorus content of the soil.<sup>63a</sup> It was suggested that information can be obtained on the presence of available plant food in the soil by determining the food requirements of bacteria. When a mannitol solution free from phosphorus yields a good growth of *Azotobacter*, after inoculation with soil, it may be assumed that the soil is not deficient in phosphorus so far as its availability to crops is concerned. According to Stoklasa,<sup>64</sup> 5.0 to 5.7 mgm. of atmospheric nitrogen are fixed for every mgm. of phosphorus used. The minimum need of phosphorus is 2.46 mgm. of P (or 5.46 mgm.  $\text{P}_2\text{O}_5$ ) for every gram of glucose. Sulfur in the form of sulfates is essential for the growth of *Azotobacter*.<sup>65</sup>

Iron, in the form of its salts, has a definitely favorable influence upon the development of *Azotobacter*, by playing a part in its metabolism and exerting a favorable influence through its colloidal condition. When iron sulfate was added to the medium the amount of nitrogen fixed per gram of mannitol was increased from 2.23 to 10.3 mgm.<sup>66</sup> It has been suggested that the colloidal iron absorbs the nitrogen and oxygen of the air and thus brings them into more intimate contact with the cells of *Azotobacter*. In the presence of organic colloids, only small quan-

<sup>63</sup> Dzierzbicki, A. *Bul. Intern. Acad. Sci. Cracovie, B.* 1910, 21-66.

<sup>63a</sup> Reed, H. S. and Williams, B. *Va. Agr. Exp. Sta. Tech. Bul.*, 4: 81-95. 1915; Walton, J. H. *Mem. Dept. Agr. India, Bact. Ser.*, 1: 97-112. 1915.

<sup>64</sup> Stoklasa, J. *Centrbl. Bakt. II*, 29: 385-419. 1911; Christensen, H. R. *Centrbl. Bakt. II*, 43: 1-166. 1915; *Soil Sci.*, 15: 329-360. 1923; Greaves, 1918 (p. 494); Niklas, H., Scharrer, K. and Strobel, A. *Landw. Jahrb.* 63: 387. 1926.

<sup>65</sup> See also Koch, A. *Ber. deut. landw. Gesell.*, 22: 117-121. 1907; *Jour. Landw.*, 55: 355-416. 1907.

<sup>66</sup> Remy, Th. and Rösing, G. *Centrbl. Bakt. II*, 30: 349-384. 1911; 33: 618-623. 1912.

tities of iron are effective. The favorable influence of soil infusion upon nitrogen fixation by *Azotobacter* has been found to be due chiefly to their content of iron and partly to silicic acid. Inorganic colloids, like the oxides of aluminum, iron and colloidal silicic acid, greatly stimulate nitrogen-fixation by *Az. chroococcum*.<sup>67</sup> After inserting a strip of paper into the medium, *Azotobacter* grows exclusively in contact with the paper. This led Söhngen to conclude that microbial life in the soil takes place chiefly upon the colloids. Iron has the same stimulating effect upon nitrogen-fixation as humus substances do, but much larger quantities of the inorganic colloid are required when it is present alone than when it is used together with the organic colloid. The iron is most active in the form of an organic compound.

Some salts may act directly as stimulants to nitrogen-fixing bacteria. The optimum concentration of manganese for nitrogen-fixation by *Azotobacter chroococcum* in mannitol solution was found to be<sup>68</sup> 1 to 2 mgm. per 100 gram of medium; further increases diminished nitrogen-fixation. One part of manganese per million parts of soil was the optimum for *Cl. pastorianum*. The addition of small amounts of arsenic (10 parts per million) exerted a stimulating effect upon nitrogen-fixation in the soil and upon the more economic utilization of energy by *Azotobacter*.<sup>69</sup> Aluminum has been looked upon<sup>70</sup> both as a stimulant and as a retarding agent. The influence of calcium and strontium upon the catalysis of nitrogen fixation by *Azotobacter* has recently been pointed out by Burk.<sup>71</sup>

Uranium salts exert a decided stimulating effect upon *Azotobacter*.<sup>72</sup> The maximum amount of nitrogen fixed and maximum growth take place in the yellow or green rays; the least, in violet light. The maximum amount of nitrogen per gram of sugar decomposed is fixed in blue light; the least, in yellow light.

*Influence of organic matter upon nitrogen fixation.* The favorable influence of soluble soil organic matter, or humus, on nitrogen-fixation was known for a long time;<sup>73</sup> however, it was recognized that the nature

<sup>67</sup> Söhngen, N. L. Centrbl. Bakt. II, **38**: 621-647. 1913.

<sup>68</sup> Olaru, D. A. Rôle du manganèse en agriculture. Paris, Baillères. 1920.

<sup>69</sup> Greaves, J. E. Jour. Agr. Res., **6**: 389-416. 1916.

<sup>70</sup> Kaserer, H. Ber. deut. bot. Gesell., **28**: 208-262. 1910.

<sup>71</sup> Burk, D. Arch. Mikrob., **2**: 155-186. 1931.

<sup>72</sup> Kayser, E. Compt. Rend. Acad. Sci., **171**: 969-971. 1920; **172**: 183-185, 491-493, 1133-1134. 1921; Ann. Inst. Nat. Agron. (2), **16**: 11-43. 1922.

<sup>73</sup> Krzemieniewski, S. Bull. Int. Acad. Sci. Cracovie, Cl. Sci. Math. Nat., No. 9: 929-1051. 1908.

of this action depends on the source of the organic matter. It was at first suggested that some of the constituents of the soil organic matter are used by *Azotobacter* as a source of energy. Löhnis and Green,<sup>74</sup> however, working with various species of *Azotobacter* in mannitol solution, reported that the amount of nitrogen fixed in three weeks in 100 cc. of solution was 5.6 mgm.; the addition of green manure increased it to 8.0 mgm., fresh stable manure to 9.8 mgm., and fresh straw to 10.0 mgm. When these substances were previously decomposed, the fixation of nitrogen was even greater. By increasing the amount of stable manure added up to three per cent, there was<sup>75</sup> a great increase in nitrogen-fixation. The stimulating effect is probably caused by the increase in available energy, due to the introduction of straw and its derivatives, and by the colloidal content of the material. A further increase in organic content may bring about a depression unless the soil is well aerated and contains sufficient  $\text{CaCO}_3$ . The various plant residues, such as roots, leaves and stems will react similarly. The possible favorable influence of growth-promoting substances upon *Azotobacter* is still a subject of discussion.<sup>76</sup>

The beneficial action of humus is frequently ascribed to its inorganic constituents, particularly aluminum and silicic acid. This is confirmed by the fact that the so-called artificial humus has no such effect, while the source of the natural humus influences the degree of its beneficial action. The claim that the action of the humus is due to its inorganic constituents has been further substantiated by the fact that purified humates do not possess the stimulating effect. The rôle of the colloid is probably chiefly due to its catalytic action and its protective action against poisons; the protective action of the colloid has also been ascribed to the distribution of the phosphorus and to the buffering effect upon the reaction of the medium.<sup>77</sup>

<sup>74</sup> Löhnis, F. and Green, H. H. *Centrbl. Bakt.* II, **41**: 52-60. 1914.

<sup>75</sup> Hanzawa, 1913 (p. 505); Tottingham, W. E. *Jour. Biol. Chem.*, **24**: 221-225. 1916.

<sup>76</sup> Bottomley, W. B. *Proc. Roy. Soc. B.*, **82**: 627. 1910; **85**: 466. 1912; **88**: 237-247. 1914; **89**: 102. 1915; *Ann. Bot.*, **28**: 531-540. 1914; Mockeridge, F. A. *Proc. Roy. Soc. B.*, **89**: 508-533. 1915; *Biochem. Jour.*, **19**: 272-283. 1915; Allen, E. R. *Ann. Mo. Bot. Gard.*, **6**: 1-44. 1919; Itano, A. *Jour. Bact.*, **8**: 483-486. 1923; Hunter, O. W. *Jour. Agr. Res.*, **23**: 825-830. 1923; Olsen, C. *Compt. Rend. Lab. Carlsberg*, **18**: 1-16. 1930.

<sup>77</sup> Kaserer, H. *Centrbl. Bakt.* II, **31**: 577-578. 1912; Voicu, J. *Compt. Rend. Acad. Sci.*, **175**: 317-319. 1922.

It was shown more recently<sup>78</sup> that while "humic acid" increases the rate and efficiency of nitrogen fixation and decreases the limiting available nitrogen pressure, it is not directly concerned in the chemical mechanism of fixation. The organic matter was also believed to take a part in the assimilation of the nitrogen rather than in its fixation.

According to Voicu, organic matter influences the sensitiveness of *Azotobacter* to poisons such as boron. Urea, glycoll, formamide and allantoin depress nitrogen-fixation; this was attributed,<sup>63a</sup> not to a direct toxicity but to the fact that those substances furnish an available nitrogen source. Among the substances acting injuriously upon nitrogen-fixing organisms, we find caffeine, alloxan, betaine, trimethyl-amine, legumin, cinnamic acid, aspartic acid, hippuric acid, creatine, creatinine, xanthine and hypoxanthine. The first two have a stimulating effect in dilute solutions.

*Influence of reaction upon the growth of non-symbiotic nitrogen-fixing bacteria.* Lime exerts such a favorable influence upon the activities of *Azotobacter* in the soil that Christensen<sup>79</sup> suggested using the presence of this organism as an index of the lime requirement of the soil. When the reaction of the soil is more acid than pH 5.7, *Azotobacter* is absent and the soil needs lime; when the pH is above 7.4, the soil does not need any lime. But when the pH of the soil is between 5.8 and 7.3, an *Azotobacter* test is made. A definite weight of soil (5 or 10 grams) is added to a definite amount of mannitol solution free from calcium carbonate (50 or 100 cc.); the flasks are sterilized, inoculated with a fresh culture of *Azotobacter* and incubated. The greater the lime or buffer content of the particular soil, the more abundant is the growth of *Azotobacter*. The amount of pellicle development is an index of the buffer action of the soil and can yield information on its lime requirements. Out of 100 soils used, the *Azotobacter* test for the lime requirement agreed in 90 per cent of the cases with the known soil condition, while the ammonium chloride and litmus tests agreed only in 50 and 40 per cent of cases respectively. The amount of  $\text{CaCO}_3$  which should be added to the soil, to obtain maximum nitrogen fixation, varies with the soil. This is due to differences in the buffer content of soils, in

<sup>78</sup> Burk, D. Jour. Phys. Chem., **34**: 1195-1209. 1930; Iwasaki, K. Biochem. Ztschr., **226**: 32-46. 1930.

<sup>79</sup> Christensen, 1907 (p. 506); 1915 (p. 507); Soil Sci., **11**: 115-178. 1917; Intern. Mitt. Bodenk., **13**: 111-146. 1923; Centrbl. Bakt. II, **29**: 347-380. 1911; Petersen, E. J. Tidskr. Planteavl. **31**: 243-337. 1925; Niklas, H. and Poschenrieder, H. Centrbl. Bakt. II, **71**: 251-284. 1927.

addition to differences of the initial reaction. Soils of a different buffer content, even of the same initial reaction, require different quantities of lime to bring them to the same optimum reaction.

*Azotobacter* is capable of existing in many soils which contain none or only mere traces of  $\text{CaCO}_3$  and also in some soils reacting acid by the ordinary test.<sup>80</sup> A reaction equivalent to about pH 6.0 is found to be, in most cases, the limiting acid reaction for *Azotobacter*. The optimum reaction for nitrogen fixation by *Azotobacter* in pure culture seems to be closely associated with the optimum reaction for growth. Different

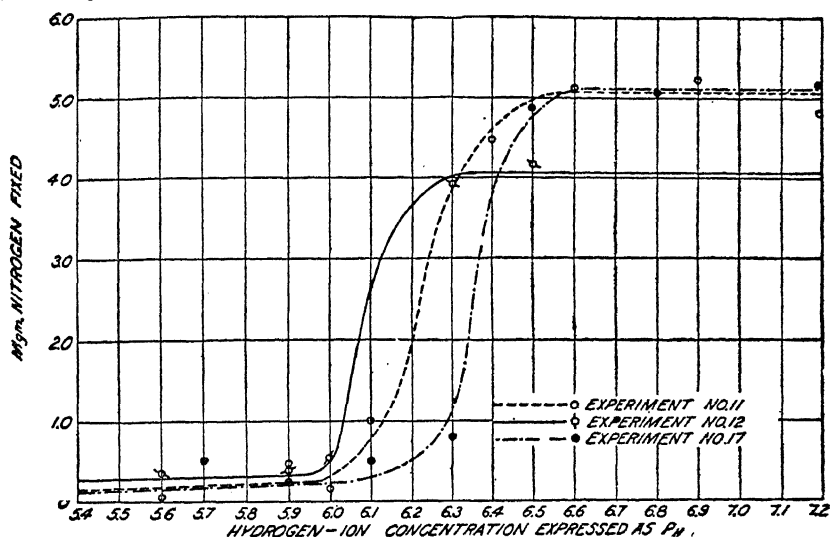


FIG. 39. Influence of reaction of medium upon nitrogen-fixation by *Azotobacter* (from Gainey and Batchelor).

strains of *Azotobacter* may vary in their sensitiveness to the limiting acid reactions, the minimum for growth having been reported to be in some cases pH 6.6 to 6.8.<sup>81</sup> The optimum reaction for the development of *Azotobacter* is pH 7.0 to 7.8, while the limiting alkaline reaction was reported to be pH 8.8.<sup>82</sup> Different species of *Azotobacter* may vary, however, also in their behavior to the optimum reaction.<sup>83</sup>

<sup>80</sup> Gainey, P. L. *Science*, N. S., 48: 139-140. 1922; *Jour. Agr. Res.*, 14: 265-271. 1918.

<sup>81</sup> Fred, E. B. and Davenport, A. *Jour. Agr. Res.*, 14: 317-336. 1918.

<sup>82</sup> Johnson, H. W. and Lipman, C. B. *Univ. Cal Publ. Agr. Sci.*, 4: 397-405. 1922.

<sup>83</sup> Yamagata and Itano, 1923 (p. 112).



The fixation of nitrogen in soils of a greater acidity than the limiting reaction for *Azotobacter* (pH less than 6.0) is due to the activities of *Cl. pastorianum*, which has its optimum at pH 6.9 to 7.3, but can still grow at an acidity greater than pH 5.7.<sup>84</sup> Growth of this organism can be obtained at as low a pH as 5.0. Other nitrogen-fixing forms, like *Bact. aerogenes* and *Radiobacter*, can also grow at a higher acidity than *Azotobacter*. The nitrogen fixed under these conditions is much less than that fixed in soils supporting an *Azotobacter* flora. Fig. 39 shows the correlation between the hydrogen-ion concentration and nitrogen fixation by *Azotobacter*.<sup>85</sup>

*Influence of moisture and temperature upon nitrogen-fixation.* Nitrogen-fixing bacteria are able to resist drying for a long period of time, depending upon the nature of the medium. The soil contains substances which exert a protective influence upon bacteria subjected to desiccation; bacteria resist desiccation longer in a rich clay soil than in sand, probably because of the colloidal content of the clay.<sup>86</sup> Nitrogen-fixation takes place in the soil even when its moisture content is very low. Soil with a higher content of organic matter will have a higher moisture optimum. An excess of water may stop the action of *Azotobacter* but may stimulate anaerobic bacteria.

Using a loam soil with a maximum moisture holding capacity of 27.4 per cent, Traaen<sup>87</sup> observed a fixation of 1.9 mgm. of nitrogen in 100 grams of soil with 5 to 10 per cent moisture, 13.2 mgm. with 17.5 per cent moisture, 16.6 mgm. with 25 per cent moisture, and 15.5 mgm. with 30 per cent moisture. At a temperature of 13°C., the amounts of nitrogen fixed were less, with a similar maximum. With the higher moisture content, the anaerobic organisms play an important part in the fixation of nitrogen. Two maxima for nitrogen-fixation in relation to the water content of the soil are frequently recorded, depending on whether the conditions are favorable to the action of aerobic or anaerobic bacteria.<sup>88</sup>

The optimum temperature for the activities of nitrogen-fixing bacteria

<sup>84</sup> Dorner, 1924 (p. 172).

<sup>85</sup> Gainey, P. L. and Batchelor, H. W. Jour. Agr. Res., 24: 759-768. 1923; Lipman, C. B. and Burgess, P. S. Jour. Agr. Sci., 6: 484-494. 1914; Gainey, P. L. Jour. Agr. Res., 24: 185-190. 1923.

<sup>86</sup> Giltner, W. and Langworth, H. V. Jour. Agr. Res., 5: 927-942. 1916.

<sup>87</sup> Traaen, A. E. Centrbl. Bakt. II, 45: 119-135. 1916.

<sup>88</sup> Lipman, C. B. and Sharp, L. T. Bot. Gaz., 59: 402-406. 1915; Greaves, J. E. and Carter, E. G. Jour. Agr. Res., 6: 889-926. 1916; also 9: 293-341. 1917.

lies at 28°C. (25° to 30°), with limits between 9° and 33°C.<sup>89</sup> Koch<sup>90</sup> obtained fixation of 3, 11, and 15.5 mgm. of nitrogen in 100 grams of soil at 7°, 15°, and 24° respectively. The maximum temperature for *Azotobacter* is 55° to 60° and the minimum is near zero. The organism can withstand heating at 45° to 50°C. for 15 minutes, but is destroyed in 30 minutes. The optimum temperature for the growth of *Cl. pastorianum* is 20° to 30°C. At 30° to 35°, the action of the organism is retarded.<sup>91</sup> The cell can withstand a temperature of 75° for 5 hours or more; the spores can be preserved in a dry state for 20 years, without losing their power of germination and nitrogen fixation.

*Soil cultivation and nitrogen-fixation.* It has been generally observed<sup>92</sup> that fallowing leads to an increase in nitrogen-fixation, probably due to better aeration and moisture conditions. According to Hiltner,<sup>93</sup> non-symbiotic nitrogen-fixation is stimulated by growing plant roots; the higher plants use up the available nitrogen in the soil and thus create a nitrogen-hunger for the non-symbiotic nitrogen-fixing bacteria. The plants supply the bacteria with available energy, in the form of rotting roots hairs, root tips, etc. Plant roots may also create a better physical environment for the nitrogen fixing organisms. In view of the fact that different cultural methods are used for the growth of different crops, the influence of the crops upon nitrogen-fixation will vary. A much higher nitrogen-fixing power was found<sup>94</sup> in cultivated than in virgin soils; the fallowed soils show more nitrogen fixed than the cropped soils.

*Importance of non-symbiotic nitrogen-fixation in soil.* It should not be assumed that the addition of available carbohydrates to various soils is always sufficient to induce non-symbiotic fixation of nitrogen. In the presence of available nitrogen in the soil, the addition of carbohydrates stimulates the development of various fungi and bacteria which use the added source of energy and transform the available nitrogen into microbial protein; under these conditions, even the non-symbiotic nitrogen-fixing bacteria act upon the carbohydrates like other heterotrophic bacteria, merely synthesizing proteins. Only in the absence of

<sup>89</sup> Krzemieniewski, 1908 (p. 508); Löhnis and Westermann, 1908 (p. 111).

<sup>90</sup> Koch, A. Ber. deut. landw. Gesell., 22: 117-121. 1907.

<sup>91</sup> Omeliansky, W. L. Arch. Sci. Biol. Petrograd, 19: 209-228. 1915.

<sup>92</sup> Heinze, B. Landw. Jahrb., 35: 889-910. 1906.

<sup>93</sup> Hiltner, L. Arb. deut. landw. Ges. H. 98: 59-78. 1904 (Centrbl. Bakt. II, 14: 46-48. 1904).

<sup>94</sup> Greaves, J. E. Centrbl. Bakt. II, 41: 444-459. 1914; Reed, H. S. and Williams, B. Va. Agr. Exp. Sta. Tech. Bul., 3: 59-80. 1915.

available nitrogen is there a probability of nitrogen fixation by non-symbiotic bacteria. But even when fixation of nitrogen takes place the process is usually a slow one in normal soils; in many cases, the actual amount of nitrogen fixed falls within the probable error for the determination of total nitrogen.

There are certain claims in the literature that very porous soils of a moderately high water content can fix small amounts of nitrogen under sterile conditions.<sup>95</sup> There is still more definite evidence that appreciable quantities of nitrogen can be fixed both in the laboratory and in the field by non-symbiotic bacteria, when there is sufficient available energy.<sup>96</sup> Remy found that considerable nitrogen fixation takes place as long as provision is made for the neutralization of the acids formed and a proper source of energy is present. The nitrogen fixed by the bacteria becomes a proper source of nitrogen for higher plants; it becomes available slowly, although not less so than the most active organic fertilizers. It has been suggested<sup>97</sup> that the great economy with which the nitrogen fixing bacteria use the organic matter in the soil is due to their symbiotic action with algae. There is no doubt that the nitrogen content of sand or soil may be appreciably increased by the activity of *Azotobacter*, if sufficient energy is supplied.<sup>97</sup> About 6 mgm. of nitrogen were fixed per 1 gram of plant residue, under laboratory experiments, and up to 9 mgm. in pot experiments.<sup>98</sup>

Among the most important conditions required for non-symbiotic nitrogen fixation are: (1) a proper supply of energy material, (2) sufficient  $\text{CaCO}_3$  to neutralize soil acidity and improve the physical condition of the soil, (3) available phosphorus, (4) proper temperature, and (5) aeration of the soil. According to statements usually made in the texts and found at Rothamsted and other stations, non-symbiotic nitrogen-fixing bacteria add, under favorable conditions, 15 to 40 pounds of available nitrogen to each acre of soil yearly and usually not more than 10 pounds. In view of the fact that the energy added to the soil is not directly available to the nitrogen-fixing bacteria, that small amounts of available nitrogen are always present in the soil, and that the

<sup>95</sup> Warmbold, H. *Landw. Jahrb.*, **35**: 1-123. 1906. *Centrbl. Bakt.* **II**, **20**: 121-126. 1907.

<sup>96</sup> Koch, A., Litzendorff, J., Krull, F. and Alves, A. *Jour. Landw.*, **55**: 355-416. 1907; **57**: 269-286. 1909; Remy, Th. *Centrbl. Bakt.* **II**, **22**: 561-651. 1909; Löhnis, F. *Centrbl. Bakt.*, **15**: 361. 1905; Schneidewind. *Ibid.*, **21**: 437. 1908; Fischer, H. *Ibid.*, **22**: 654. 1909.

<sup>97</sup> Krainsky, A. *Centrbl. Bakt.* **II**, **26**: 231-235. 1910.

<sup>98</sup> Hutchinson, H. B. *Jour. Agr. Sci.*, **9**: 92-111. 1918.

error in the laboratory determination of total nitrogen by the Kjeldahl method is greater than the possible amount of nitrogen fixed by non-symbiotic bacteria, we are still unable to decide the question definitely. The field results of A. Koch do not speak for any nitrogen fixation in the soil, following the addition of cellulose and even straw.<sup>99</sup> Positive fixation was obtained only when soluble sugars were added, as seen from table 49.

TABLE 49

*Influence of sugar upon crop yield and nitrogen content of crop*

YEAR	CONTROL	GRAMS OF DRY SUBSTANCE PER POT FOR 3-YEAR PERIOD		
		Glucose, 360 grams	Cane sugar, 360 grams	Cane sugar, 720 grams
1905-07	91.3	111.6	113.2	157.6
1908-10	51.2	78.5	77.6	91.8
1911-13	76.1	79.8	85.6	89.9
1914-16	63.6	65.9	68.6	67.4
1917-19	73.0	80.9	75.6	69.6
1920-22	65.3	64.0	72.6	66.3
Total.....	420.5	480.7	492.2	542.6
Excess over control.....	....	60.2	72.7	122.1
		MILLIGRAMS OF NITROGEN IN CROP		
1905-07	765	984	982	1476
1908-10	519	785	808	883
1911-13	616	634	670	739
1914-16	463	613	540	548
Total.....	2363	2916	3000	3640
Excess over control.....	....	553	637	1283

By determining the amount of nitrogen fixed in soil per gram of sugar added, it was found that, although 720 grams of cane sugar had been added per pot of soil and 9.75 mgm. of nitrogen had been fixed per gram of sugar added, the plants utilized only about one-fifth of the nitrogen fixed. The larger part remained in the soil in a complex form not readily utilized. The addition of cellulose exerted a decided injurious effect upon crop yield due to competition for the available nitrogen between the microorganisms and the plants. The following years the nitrogen was

<sup>99</sup> Rippel, A. Jour. Landw., 72: 17-52. 1924.

TABLE 50  
*Influence of cellulose upon crop yield*

YEARS	CONTROL	GRAMS OF DRY SUBSTANCE PER POT FOR 3-YEAR PERIOD		
		120 grams paper	120 grams paper + manure infusion	Manure infusion alone
1911-13	68.3	12.8	17.9	67.0
1914-16	60.0	81.7	87.3	62.8
1917-19	66.0	77.4	82.8	69.9
1920-21	65.2	71.1	72.8	67.0
Total.....	259.5	243.0	260.8	266.7

made available again; however, one cannot speak here of any nitrogen fixation. Similar results were obtained with straw (table 50).

#### SYMBIOTIC NITROGEN FIXATION

*Relation between the bacteria and the host plant.* "Virulence" in connection with nodule bacteria has been defined as the ability of the organism to penetrate into the root tissues of the host plant, to multiply there, and to cause a certain benefit or injury. Various attempts have been made to study this physiological property by vegetation experiments. Hiltner<sup>100</sup> observed an increased growth of leguminous plants (peas) when grown continuously upon the same soil; he ascribed this not only to an increase in the number of bacteria causing inoculation, but also to an increase in the virulence of the bacteria, similar to an increase in virulence of a pathogenic organism when passed through several animals. Assuming that the nodule bacteria increase in virulence by repeated symbiosis with plants, Hiltner planted peas repeatedly on the same soil, which was sufficiently provided with minerals; he found an increase in the infection by the organism from the first to the fourth generation, a period without change then followed, and finally the continued growth of peas gradually led to a diminution in plant growth.

On the basis of these results, Hiltner proposed the "immunity" theory, according to which substances are formed by the bacteria within the nodules which immunize the plant against further invasion of bacteria. The organism (1) may not penetrate into the plant, (2) it may gain admission, but without producing nodules because of the greater resistance of the plant, (3) it may enter the plant and produce nodules but without fixing any nitrogen, (4) it may fix nitrogen which is assimilated

<sup>100</sup> Hiltner, 1904 (p. 513).

by the plant, (5) the bacterium may become more efficient than the plant, which is then injured. According to the "immunity" theory, active nodules impart to the plant an immunity against bacteria of lower or equal virulence than those already found in the plant; only bacteria of high virulence are capable of penetrating into the plant. The above theory was not confirmed by subsequent investigations. Nodules are transient on biennial and perennial legumes, depending somewhat on the climatic conditions; i.e., there are two crops of nodules in biennial legumes, one each year, while there are many crops on perennial legumes such as alfalfa. When a fresh culture is added to a leguminous plant growing on agar and having already formed nodules, more nodules are produced on the new roots that have grown since the first inoculation. Under these conditions, one cannot speak of plant immunity against further invasion by bacteria.<sup>101</sup>

The "equilibrium" theory proposed by Süchting,<sup>102</sup> as an explanation of the mutual relationship between the leguminous plant and nodule-forming bacteria, is more plausible and has many facts to support it. A state of equilibrium was considered to exist between the attacking power of the bacteria and the resisting power of the plant, due perhaps to the fact that the bacteria produce a toxin and the plants an antitoxin. The degree of equilibrium determines the extent of nodule formation, the plant becoming immunized by an antibody and not by a substance produced by the bacteria; the nitrogen supply is regulated by the production of the antibody. When the leguminous plants are grown in soil containing a sufficient supply of nitrates, their resisting power to the infection of the bacteria is greater than when grown on nitrogen free media. The bacteria may vary in virulence, depending on the media in which they are grown. Increasing virulence was also found to be directly correlated with a shortening of the vegetation period of the plant.<sup>103</sup>

The nitrogen is fixed by the bacteria present within the nodules and is made available for the growth of the host plants by the autolysis of these nodules, or through the production of bacteriolytic enzymes by the plant.<sup>104</sup> The plant obtains its carbon from the CO<sub>2</sub> of the atmosphere by photosynthetic processes; a part of the carbohydrates thus

<sup>101</sup> Whiting, A. L. Ill. Agr. Exp. Sta., Bul. 179. 1915.

<sup>102</sup> Süchting, H. Centrbl. Bakt. II, 11: 377-388, 417-441, 496-520. 1904; Hocquette, M. Compt. Rend. Acad. Sci., 191: 1363-1365. 1930.

<sup>103</sup> Pfeiffer, H. Centrbl. Bakt. II, 73: 28-57. 1928.

<sup>104</sup> Grijns, A. Centrbl. Bakt. II, 60: 248-251. 1927.

synthesized is transferred to the roots and is used by the bacteria as a source of energy.

Wunschik<sup>105</sup> based his idea of the relation between the bacterium and the host plant on the statement of Beijerinck that "when living plant cells have to derive help from another organism, an equilibrium between the growth of both must be reached." The equilibrium is in this case established between the vegetative energy of the plant and of the nodule forming organism. Wunschik differentiated between the vegetative energy, or ability to penetrate into the roots of the plant, and nitrogen-fixing capacity of the organism. The vegetative energy of the bacteria results in the removal from the host plant of a part of its nutrients, thus causing injury; the nitrogen-fixation by the bacterium is beneficial to the plant and is, to a certain extent, correlated with the vegetative energy of the bacterium. This stimulates the growth energy of the plants. When the equilibrium stage is reached, the growth of the plant continues uninterrupted. The vegetative energy of the nodule bacteria is increased by repeated physiological adaptation to the host plant, namely by repeated passage through the plant.

*Chemistry of nitrogen-fixation by symbiotic bacteria.* In the presence of an abundance of available nitrogen in the soil, the leguminous plants utilize that nitrogen and do not depend on the activities of the bacteria.<sup>106</sup> Alkali nitrates in concentrations of 1:10,000 and ammonium salts in 1:2,000 repress nodule formation.<sup>107</sup> The addition of 5 mgm. nitrogen as  $KNO_3$  per liter of medium was sufficient to prevent the penetration of the bacteria into the roots of the plants in water cultures; this action was much less in sand and hardly obtained in soil. In some cases small amounts of nitrogenous substances were found to stimulate plant growth and nodule formation.<sup>108</sup>

The average amount of nitrogen fixed by a good crop of a legume, under favorable conditions, may be taken as 200 pounds per acre. If the energy (carbon source) need of the organisms is 100 times the amount of nitrogen fixed, as in the case of the non-symbiotic bacteria, the symbiotic bacteria would require 20,000 pounds of carbohydrate per acre for the fixation of the favorable amount of nitrogen, which would have to be supplied by the growing plant. This would be equivalent to between two and four times as much as the total crop yield of the

<sup>105</sup> Wunschik, 1925 (p. 133).

<sup>106</sup> Wohltmann and Bergené. Jour. Landw., 50: 377-395. 1902.

<sup>107</sup> Marchal, E. Compt. Rend. Acad. Sci., 133: 1032-1033. 1901.

<sup>108</sup> Hiltner, L. Arb. biol. Anst., K. Ges. Amt., 11: 177-222. 1900.

plant. We must assume that the organism uses the energy supplied by the plant much more efficiently than the non-symbiotic bacteria or that the process of nitrogen-fixation by the legume bacteria is exothermic.<sup>109</sup> In the second case, the energy liberated is so small that it would hardly be sufficient to cover the need of the bacteria for metabolism alone. It may then be supplemented by some of the carbohydrates synthesized by the plant.

Of the three possible processes by which the nitrogen can be fixed, namely, (a) reduction, (b) oxidation and (c) direct union with organic compounds, the first is the most plausible, especially in view of the fact that a great many microorganisms assimilate the nitrogen in the form of ammonia. Whiting<sup>110</sup> could not demonstrate any ammonia, nitrites or nitrates within the plant; this tends to give weight to the direct organic combination theory. Some evidence was previously obtained<sup>111</sup> concerning the possibility of a direct union between the free nitrogen and some organic compound inside the bacterial cell; it was believed that glycogen and carbamic acid are the first products of combination. It may be of interest to note here that the nodule bacteria are capable of producing in pure culture pyruvic acid from sugars.<sup>112</sup>

Once the nitrogen has been fixed in the bacterial cells, it may be transferred to the host when these cells (so-called bacteroids) are decomposed and the contents absorbed by the plants, or when the nitrogen has been secreted by the bacterial cells in a form which the plant then utilizes.<sup>113</sup> This removal of the products of bacterial growth by the plant was believed to stimulate further nitrogen fixation. The chemical nature of the nitrogenous substance in the bacterial secretions is still unknown, except that it is believed to be of the nature of amino acids, since these compounds are readily assimilated by the leguminous plants.<sup>114</sup>

It has been demonstrated<sup>115</sup> that the nodules of some leguminous

<sup>109</sup> Christiansen-Weniger, 1923 (p. 500); Allam, F. *Ztschr. Pflanzen. Düng. A.* 20: 270-301. 1931.

<sup>110</sup> Whiting, A. L. and Schoonover, W. R. *Soil Sci.*, 10: 411-420. 1920.

<sup>111</sup> Gerlach and Vogel, 1903 (p. 365); Heinze, B. *Landw. Jahrb.*, 35: 889-910. 1906.

<sup>112</sup> Anderson, J. A., Peterson, W. H. and Fred, E. B. *Soil Sci.*, 25: 123-132. 1928.

<sup>113</sup> Nobbe and Hiltner, 1900 (p. 122); Golding, J. *Jour. Agr. Sci.*, 1: 59-64. 1905.

<sup>114</sup> Virtanen, A. I. and von Hausen, S. *Biochem. Ztschr.*, 232: 1-14. 1931; *Ztschr. Pflanzen. Düng. A.* 21: 57-69. 1931.

<sup>115</sup> Gerretsen, F. C., Grijns, A., Sack, J. and Söhngen, N. L. *Centrbl. Bakt. II*, 60: 311-316. 1923.



plants may contain a bacteriophage, which dissolves the bacteria thus making their contents available to the plant. The bacteriophage was found not only in the nodules but also in the roots and stems of the plants, but not in the leaves; also in garden and field soils, but not in prairie soils. It is specific in its lytic action and attacks only those bacteria which form the nodules in the roots of the specific plants. The bacteriophage can resist, according to species, a temperature of 60° to 65°C., for fifteen minutes. It withstands drying and passes through a thin collodium membrane; it also resists ultra-violet light eight times as much as the corresponding bacteria. To obtain the bacteriophage, fresh nodules, previously sterilized on the surface, are ground up and are placed in a nutrient medium. After 5 days, the turbid solution is filtered through a Chamberland filter and a few cubic centimeters of the clear filtrate are added to a fresh medium previously inoculated with a corresponding pure culture of the nodule organism. This is repeated after 10 days, diminishing every time the amount of liquid used for infection. This results in an accumulation of the bacteriophage, and if a few cubic centimeters of such a culture are added to a culture of *Bact. radiculicola*, the turbid culture of the latter will become transparent due to the dissolution of the bacteria. When some of the bacteriophage is placed upon an agar slant and the agar inoculated with the nodule organism, the latter will grow only where the bacteriophage is absent. Grijns<sup>116</sup> has shown that clover plants grown in sterile culture or in the presence of a non-lysogenic strain of *Bact. radiculicola* did not produce any bacteriophage. Its presence was not essential for limiting the size of the root nodules. The growth of the plant with a bacteriophage was just as satisfactory as without.

It is also possible that the plant produces bacteriolytic enzymes, which hydrolyze the bacterial cell, liberating the available nitrogen. The bacterium itself seems to produce a cellulose-dissolving enzyme, by means of which it enters the root hairs of the host plant, dissolving the cell wall;<sup>117</sup> however, this still needs confirmation.

*Production of gum by the nodule bacteria.* In artificial cultures, *Bact. radiculicola* produces a gum which goes partly into solution and is partly held by the zooglyphic masses of the organism. To prepare this gum, the bacteria are grown on synthetic media; the cells are removed by centrifuging and the gum precipitated from the solution by alcohol, acetone, concentrated solutions of ammonium sulfate, magnesium sulfate or am-

<sup>116</sup> Grijns, A. Centrbl. Bakt. II, 71: 248-251. 1927.

<sup>117</sup> Hiltner, L. Centrbl. Bakt. II, 6: 273-281. 1900.

moniacal lead acetate. The cells of the bacteria contain 53.8 per cent carbon and 4.69 per cent nitrogen; however, the gum contains 38.8 per cent carbon and no nitrogen.<sup>118</sup> The gum does not reduce Fehling's solution, but, on heating with a dilute solution of sulfuric acid (2 per cent) at 120°C. for one hour, reducing sugars are formed, indicating that it is of a hemicellulose nature. The hydrolysate was found to contain 4.1 to 25.3 per cent uronic acid, 4.3 to 16.4 per cent pentosan and a considerable amount of glucose. The gum is formed with various sources of energy in the medium, such as cane sugar, glycerol, or legume extract,<sup>119</sup> and should be considered as a synthesized product. The bacteria of the monotrichous type do not produce as much gum as those of the peritrichous type, while different strains of the same organism vary greatly in the amount of gum formed.<sup>120</sup> A further study of the nature of this substance and its rôle in the fixation of nitrogen is desirable.

*Mechanism of nitrogen fixation by leguminous plants.* The mechanism of nitrogen-fixation by the leguminous plants was a subject of early controversy. It was shown conclusively<sup>121</sup> that these plants (cowpea and soybean) fix atmospheric nitrogen through their roots and not through their leaves, as it had been assumed in some cases. In the earlier phase of the growth of the plant, the roots contain the larger part of the nitrogen; at the time of harvest, however, 74 per cent of the nitrogen of cowpeas and soybeans is found in the tops. The fixation of the nitrogen takes place in the early stages of growth of the seedling, sometimes within the first fourteen days.

A study of the composition of leguminous plants<sup>122</sup> established the presence of various amino acids and amides. Inoculation increases the protein content of the plant, often without even increasing the crop yield. Plants depending largely upon the bacteria for their nitrogen show a high alkaloid content; plants which obtain their nitrogen from inorganic nitrogenous compounds, especially lupines, are poor in alkaloids.<sup>123</sup>

<sup>118</sup> Hopkins, E. W., Fred, E. B. and Peterson, W. H. Jour. Bact., **15**: 22. 1928; **17**: 22. 1929; Jour. Amer. Chem. Soc., **52**: 3659-3668. 1930.

<sup>119</sup> Buchanan, R. E. Centrbl. Bakt. II, **22**: 371-396. 1909; Grieg-Smith, R. Centrbl. Bakt. II, **30**: 552-556. 1911; Fred, 1911-12 (p. 122).

<sup>120</sup> Burrill and Hansen, 1917 (p. 121); Wright, 1925 (p. 122).

<sup>121</sup> Whiting, 1915 (p. 517).

<sup>122</sup> Schulze, E. Ztschr. physiol. Chem., **24**: 18-114. 1895; **30**: 241-312; **48**: 387, 396. 1906.

<sup>123</sup> Weber, E. Inaug. Diss. Leipzig. 1920.

Table 51 shows that inoculation results in a large increase in the nitrogen content of the plant, but this is accompanied by a still larger increase in its alkaloid content. When sterile plants obtain their nitrogen from  $(\text{NH}_4)_2\text{SO}_4$  or  $\text{NaNO}_3$ , not only is the alkaloid content lower, but the nitrogen content of the plant decreases accordingly.

*Importance of symbiotic-nitrogen fixation in the soil.* It has been shown previously that, in the case of non-symbiotic nitrogen fixation, the evidence as to actual amount of nitrogen fixed under field conditions is still of doubtful value. In the case of symbiotic fixation of nitrogen, the evidence is undisputed. The amount of nitrogen added to the soil by the bacteria depends upon the relative abundance of available nitrogen in the particular soil, both in inorganic and organic forms. The poorer

TABLE 51

*Influence of inoculation and fertilization upon yield, alkaloid and nitrogen content of Lupinus angustifolius*

TREATMENT	YIELD OF GRAIN			YIELD OF STRAW	
	Weight	Alkaloid content	Nitrogen content	Weight	Nitrogen content
Sterile, uninoculated.....	100	100	100	100	100
Sterile, inoculated (average of two good preparations).....	244	1648	554	119	266
Sterile, fertilized with $(\text{NH}_4)_2\text{SO}_4$ ..	186	558	229	111	113
Sterile, fertilized with $\text{NaNO}_3$ .....	200	678	236	113	103
Unsterilized, uninoculated.....	151	738	300	56	66
Unsterilized, inoculated.....	144	577	277	59	81

the soil is in available nitrogen (for the growth of the leguminous plants) and the richer it is in lime, available phosphorus and potash, the greater will be the gain in nitrogen. In addition to this, the kind of legume and seasonal conditions affect the amount of nitrogen fixed. The maximum amount of nitrogen was found to be fixed a little before, or just at blossoming time.

Warington<sup>124</sup> pointed out in 1891 that an approximate increase of 350 pounds of nitrogen per acre may be obtained as a result of the growth of inoculated legumes (clover). Since then, extensive data have been secured, all of which point to definite increases in soil nitrogen due to the growth of leguminous plants in the presence of the proper bacteria. Poor soils are usually found to give larger gains than rich soils. Soils to

<sup>124</sup> Warington, R. U. S. Dept. Agr. Off. Exp. Sta. Bul. 8, 22-41. 1892.

which lime and phosphorous compounds have been added show greater increases in combined nitrogen than soils where those minerals were lacking. Inoculated soils give better results than uninoculated, particularly if the legume in question or the related forms have not been grown previously on the same soil. Hiltner,<sup>125</sup> for example, obtained an increase of 1.7 to 31 times the yield for lupines and 15-80 times for serradella as a result of inoculation with the proper organism. On the average, there may be a gain of 50 to 100 pounds of nitrogen per acre of soil due to the growth of legumes. Lipman and Blair<sup>126</sup> found a gain of 54 pounds annually over a period of seven years from the growth of legumes in rotation with corn, potatoes, oats and rye in cylinders.

According to Hopkins,<sup>127</sup> a 3-ton crop of cowpea hay adds 86 pounds of nitrogen per acre, a 25-bushel crop of soybeans with  $2\frac{1}{4}$  tons of straw adds 106 pounds, a 4-ton clover crop adds 106 pounds and a 4-ton alfalfa crop adds 132 pounds. At least two-thirds of the nitrogen in legumes grown on normally productive soils is obtained from the air. Under optimum conditions and on a relatively poor soil, as much as 400 pounds of nitrogen may be added per acre per year.<sup>128</sup> The net yearly gain per acre from the growth of clover on a light sandy soil was found to be 50 pounds of nitrogen.<sup>129</sup> From 120 to 250 mgm. of nitrogen are fixed per plant of red clover and alfalfa.<sup>130</sup> If the tops are removed, the nitrogen content of the soil may not be increased, since the amount fixed may be just sufficient to fulfill the need of the tops. In the case of perennial legumes, like alfalfa, there may not be an actual increase in soil nitrogen, as compared with uncultivated soils, although the nitrogen is higher than in the same soils upon which grains are grown.<sup>131</sup>

<sup>125</sup> Hiltner, 1904 (p. 513).

<sup>126</sup> Lipman, J. G. and Blair, A. W. N. J. Agr. Exp. Sta. Bul. 289. 1916.

<sup>127</sup> Hopkins, C. Ill. Agr. Exp. Sta. Bul. 94. 1904.

<sup>128</sup> Wheeler, H. J. R. I. Agr. Exp. Sta. Bul. 152. 1912.

<sup>129</sup> Shutt, F. T. Experiment Farms Rpt., Ottawa. 1912, 144-146.

<sup>130</sup> Brown, P. E. and Stallings, J. H. Soil Sci., 12: 365-407. 1921.

<sup>131</sup> Swanson, C. O. and Latshaw, W. L. Soil Sci., 8: 1-39. 1919.

## CHAPTER XXI

### TRANSFORMATION OF SULFUR BY MICROORGANISMS

*Sources of sulfur in soil and processes of transformation.* In addition to carbon and nitrogen, there are a number of other elements which are of prime importance in the growth of plants and microorganisms. We need only mention sulfur, phosphorus, potassium, iron, calcium and magnesium. The transformation of sulfur by microorganisms will be discussed in detail not because this element is more important than the others, but because, next to carbon and nitrogen and except for oxygen and hydrogen, it is required by the majority of organisms for structural purposes. It is also used by certain organisms for energy purposes, while certain compounds of sulfur offer favorable sources of oxygen to specific bacteria, under anaerobic conditions. Sulfur is more similar to nitrogen than any other element in the many transformations that it enters and in the variety of microorganisms which produce these transformations. One finds in the sulfur cycle apparent duplications of the processes associated with the nitrogen cycle.

Sulfur occurs in the soil and may be introduced there in the form of complex organic substances<sup>1</sup> as well as various inorganic compounds. The latter comprise elementary sulfur, sulfides and sulfates. Sulfur is a less limiting factor for plant growth than nitrogen is. Considerably larger quantities of mineralized sulfur are present in the soil than of nitrogen. Rippel<sup>2</sup> found that in forest soils, with a sulfur content of 0.058 to 0.085 per cent, the sulfate sulfur made up about 10 per cent of the total, independent of the reaction of the soil; in compost soil with 0.126 per cent sulfur, the sulfate content was 20.6 per cent of the total; in field soils with 0.042 per cent of sulfur, the sulfate was 14.4 per cent of the total. Bertrand<sup>3</sup> found that the total sulfur content of a rich fertile soil was 0.52 per cent, of which 0.11 per cent was in an inorganic or sulfate form and 0.41 per cent in an organic form. Just as in the

<sup>1</sup> Berthelot, M. *Chimie végétale et agricole.*, 4: 70, 1899; Shedd, Kentucky Agr. Exp. Sta., Bul. 32, 1922; Woodward, Bot. Gaz., 73: 81, 1922; Eaton, Ibid., 74: 32. 1922.

<sup>2</sup> Rippel, A. *Jour. Landw.*, 76: 1-10. 1928.

<sup>3</sup> Bertrand, G. and Silberstein, L. *Ann. Sci. Agron.*, 43: 71-77. 1927.

case of the organic nitrogen compounds, the organic sulfur compounds are mineralized in the soil with great difficulty.

The organic matter commonly added to the soil, in the form of various plant and animal residues, contains from 0.1 to 0.5 per cent sulfur, as shown in table 52.<sup>4</sup> The sulfur is present in the plant chiefly in the cystine group of the protein molecule. When large quantities of sulfates are present in the soil, the plant may also assimilate an excess of sulfur and contain free sulfate. Certain plants contain various volatile sulfur compounds, including certain glucosides, such as sinigrin,

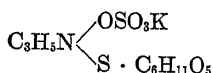


TABLE 52  
*Sulfur content of various organic materials*

MATERIAL	SULFUR
	<i>per cent</i>
Alfalfa hay.....	0.287
Barley straw.....	0.147
Oat straw.....	0.207
Rye straw.....	0.049
Wheat straw.....	0.140
Red clover.....	0.164
Corn stover.....	0.120
Cottonseed meal.....	0.487
Turnip tops.....	0.900

which is decomposed in the soil to mustard oil ( $\text{C}_3\text{H}_5\text{NCS}$ ), glucose and potassium acid sulfate.<sup>5</sup>

The sulfur content of the upper 6 to 7 inches of ordinary field soils is usually 250 to 1000 pounds per acre.<sup>6</sup> According to Kossowitsch,<sup>7</sup> the average sulfur content of the upper 30 cm. of soil is 0.1 per cent  $\text{SO}_3$ , while that of the following 70 cm. is 0.025 per cent  $\text{SO}_3$ . One-half this amount is sufficient for 285 cereal crops or 70 alfalfa crops (cereal

<sup>4</sup> Hart, E. B. and Peterson, W. H. Wis. Agr. Exp. Sta. Res. Bul., 14. 1911.

<sup>5</sup> Peterson, W. H. Jour. Amer. Chem. Soc., 36: 1290-1300. 1914.

<sup>6</sup> Woodward, J. Bot. Gaz., 73: 81-109. 1922; Olson, G. A. and St. John, J. L. Wash. Agr. Exp. Sta. Bul., 165. 1921; Joffe, J. S. N. J. Agr. Exp. Sta. Bul. 374. 1922; Olszynski, W. Roczn. Nauk. Rolnicz., 18: 231-278. 1927.

<sup>7</sup> Kossowitsch, P. C. Zhur. Opit. Agron., 14: 181-218. 1913; Johnson, E. M. Jour. Amer. Soc. Agron., 17: 589-591. 1925.

grains containing 0.29 to 0.45 per cent  $\text{SO}_3$ ; straw, 0.26 to 0.55 per cent  $\text{SO}_3$ ; alfalfa hay, 0.50 per cent  $\text{SO}_3$ ). Kossowitsch calculated that rainfall contains 1.93 to 14.17 mgm.  $\text{SO}_3$  per liter, which is equivalent to about 10 pounds per acre per year. The quantity is considerably higher near large cities. Drainage waters are richer in  $\text{SO}_3$  than rainfall, the concentration depending on climate, topography, type of soil, fertilization, etc. Sulfur is also added to the soil in the form of gypsum, superphosphates and elementary sulfur in artificial fertilizers.

Sulfur does not remain long in the soil in the form in which it is introduced. It undergoes a series of transformations involving the activities of a number of microorganisms, the specificity of which depends on the nature of the compound containing the sulfur. If the latter is introduced into the soil in the form of an organic substance, the organic matter is first decomposed by various heterotrophic bacteria, fungi and actinomyces and the sulfur bearing fraction is liberated. This is either assimilated by microorganisms or it is decomposed by various bacteria, and the sulfur is finally liberated as  $\text{H}_2\text{S}$ . That part of the sulfur which is utilized by the microorganisms for the synthesis of microbial protein has to be decomposed again before the sulfur is made available for higher plants. The  $\text{H}_2\text{S}$  is oxidized by autotrophic and facultative autotrophic bacteria to sulfur and then to sulfuric acid, which combines with the soil bases to give sulfates. The latter are either assimilated by higher plants or by microorganisms and again transformed into proteins or are reduced to  $\text{H}_2\text{S}$  by specific reducing bacteria under anaerobic conditions. The  $\text{H}_2\text{S}$  is again oxidized, under favorable conditions.

When unoxidized or partially oxidized inorganic forms of sulfur, such as thiosulfates, sulfides, including hydrogen sulfide, and elementary sulfur, are added to the soil they are at first oxidized, if the soil aeration and moisture are favorable. These substances may originate from the decomposition of organic matter in the soil, in sulfur springs, in river and sea waters, from the reduction of sulfates, from volcanic eruptions, from the burning of coal or sulfide ore smelters. The oxidation of sulfur may be both chemical and biological in nature resulting in the formation of sulfates. When soil conditions favor anaerobiosis, as in soils saturated with water, or when sufficient aeration is lacking, the sulfates as well as the elementary sulfur may be reduced to sulfides. Sulfates may be leached out from the soil into lakes and seas, where they are reduced by other microorganisms to hydrogen sulfide.

The transformation of sulfur in the soil may thus be summarized under four headings: (1) oxidation, (2) reduction, (3) synthesis (into

proteins), and (4) decomposition of proteins and protein derivatives containing sulfur.

*The nature of oxidation of sulfur and its compounds in the soil.* There is no doubt that some quantities of elementary sulfur, particularly when present in a finely divided or colloidal state, as well as small amounts of  $\text{H}_2\text{S}$  and sulfides, may be oxidized by chemical agencies, especially in the presence of proper catalysts. The oxidation of thiosulfate to tetrathionate and even to sulfate can be carried out by means of inorganic catalysts, the iodine ion and a peroxide being sufficient for the first reaction and molybdic acid and a peroxide for the second:<sup>8</sup>

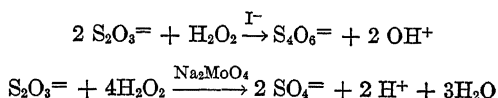
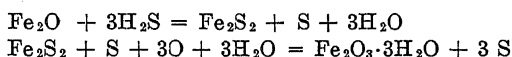


TABLE 53

*Formation of  $\text{SO}_4$  from 200 mgm. of S in 100 grams of soil in six weeks*

KIND OF SOIL	RHOMBIC SULFUR	MILK OF SULFUR
	mgm.	mgm.
Quartz sand.....	3.69	44.64
Sandy soil.....	3.81	144.22
Loam soil.....	16.45	227.58
Meadow soil.....	10.92	284.02

The purely chemical theory of sulfur oxidation has been suggested.<sup>9</sup> With quartz sand containing iron oxide, as the medium for the transformation of the sulfur used in the form of milk of sulfur or in the colloidal form, the quantities given in table 53 were oxidized. The chemical oxidation of sulfides takes place very rapidly, especially in the case of the more soluble forms;<sup>10</sup> this is believed to go through the sulfur stage, as shown by the following reactions:



<sup>8</sup> Abel, E. Ztschr. Elektrochem., 18: 705. 1912; 19: 480. 1913.

<sup>9</sup> Kappen, H. and Quensell, E. Landw. Vers. Sta., 86: 1-34. 1915; MacIntire, W. H., Gray, F. J. and Shaw, W. M. Jour. Ind. Eng. Chem., 13: 310-313. 1921; Boullanger, E. and Dujardin, M. Compt. Rend. Acad. Sci., 155: 327-329. 1912; Brioux, Ch. and Guerbet, M. Ann. Sci. Agr. (4), 2: 384-396. 1913; Compt. Rend. Acad. Sci., 156: 1476. 1913; Demolon, A. Compt. Rend. Acad. Sci. 156: 725-728. 1913.

<sup>10</sup> Brown, P. E. and Kellogg, E. H. Jour. Biol. Chem., 21: 73-89. 1915.



The elementary colloidal sulfur was rapidly oxidized to sulfate; the rhombic sulfur only very slightly. Kappen and Quensell themselves have brought forth data to demonstrate that considerably larger quantities of sulfur are oxidized in unsterile than in sterile soils. When active sulfur oxidizing organisms are present, it is easy to demonstrate that this process is primarily biological in nature.

Elementary sulfur and several sulfides are oxidized in the soil more actively by microorganisms. It was thought originally that this process is limited to certain specific groups of bacteria which are capable of utilizing the energy obtained in the process of oxidation for chemosynthetic purposes. We are coming more and more to recognize that the property of slow oxidation of sulfur or of incompletely oxidized sulfur compounds, such as sulfides, is probably widely distributed among microorganisms. Not only various common heterotrophic soil bacteria (*Bac. mycoides*, *Bact. fluorescens*) are capable of oxidizing small amounts of elementary sulfur, in nutrient solutions containing organic nitrogen and sources of energy (glycerol),<sup>11</sup> but also various common soil fungi and even actinomyces are reported as oxidizing small amounts of sulfur in artificial media and in soil.<sup>12</sup> *Pen. luteum* seems to be particularly active in this connection.

However, neither the oxidation of sulfur by chemical agencies nor its transformation by heterotrophic microorganisms can compare with the rapidity with which sulfur is oxidized, when used as a source of energy by autotrophic bacteria. The mechanism of assimilation of elementary sulfur by heterotrophic microorganisms is still unknown. It may be reduced by means of an enzyme, as in the case of yeasts,<sup>13</sup> to hydrogen sulfide and the latter assimilated and utilized for protein synthesis. The mechanism of autotrophic oxidation of sulfur has been studied in detail and is well known.

In considering the process of sulfur oxidation by autotrophic bacteria, we must differentiate carefully between the nature of the organism and the sources of sulfur. Of the different groups that have been enumerated as capable of oxidizing sulfur and its compounds (p. 77), only the *Thiobacillus* group is found in normal soils. The presence of larger forms belonging to the unbranched types, accumulating sulfur within

<sup>11</sup> Demolon, A. Compt. Rend. Acad. Sci., 173: 1408-1410. 1921.

<sup>12</sup> Abbott, E. V. Soil Sci., 16: 207-216. 1923; Rippel, A. Centrbl. Bakt. II, 62: 290-295. 1924; Guittonneau, 1926 (p. 534).

<sup>13</sup> Morison, C. B. Science, 60: 482-483. 1924; Rubner, M. Arch. Hyg., 16: 53-72. 1893.

their cells, is possible only in muds or in soils kept under anaerobic conditions, where the formation of hydrogen sulfide takes place.

The *Thiobacillus* group is present abundantly in all soils but the most active forms are found only in those soils which receive applications of sulfur as a fertilizer, in organic (sewage, etc.) or inorganic forms. This is due either to a direct introduction of the bacteria into the soil or to a response due to the addition of the specific nutrient. It was shown, for example, that soils receiving stable manure or green manure were capable of oxidizing sulfur more rapidly than the untreated soils poor in organic matter. By increasing soil aeration and keeping the moisture content at 50 per cent of the moisture holding capacity, favorable conditions are created for the oxidation of the sulfur.

TABLE 54

*Oxidation of elementary sulfur to sulfuric acid by Thiobacillus thiooxidans*

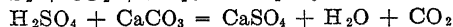
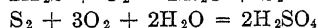
INCUBATION	AMOUNT OF CULTURE IN FLASK	CONTROL FLASK		INOCULATED FLASKS		ELEMENTARY SULFUR DISAPPEARED	* INCREASE IN SULFATE
		Sulfur	Sulfate*	Sulfur	Sulfate		
days	cc.	mgm. of S	mgm. of S	mgm. of S	mgm. of S	mgm. of S	mgm. of S
15	100	1,001	86.4	788	302.1	213	215.7
30	100	992	90.5	735	354.0	257	263.5
15	300	3,002	112.2	2,496	633.0	506	520.8
30	300	2,997	126.5	1,974	1,168.0	1,023	1,041.5

\* Milligrams of soluble sulfates as sulfur in flask; averages of 3 flasks are given; the concentration of sulfates in the small flasks was greater, due to the fact that, in these, a medium containing 2 gm.  $(\text{NH}_4)_2\text{SO}_4$  and 0.5 gm.  $\text{MgSO}_4$  per liter was used.

Oxidation of sulfur in soil may be followed by (1) an increase in acidity, as expressed by a change in the pH; (2) an increase in the amount of sulfates in the soil; (3) the disappearance of elementary sulfur. The last condition can be determined by extracting the residual sulfur from the soil with acetone.<sup>14</sup>

*Oxidation of sulfur by microorganisms.* The biological oxidation of sulfur has been studied in detail by Winogradsky.<sup>15</sup> As a source of sulfur  $\text{H}_2\text{S}$  was used or conditions were made favorable for its production.

The reactions involved in the process were presented as follows:

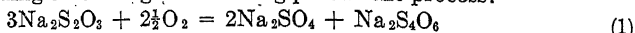


<sup>14</sup> Simon, R. H. and Schollenberger, C. J. Soil Sci., 20: 393-396. 1925; Lorant. I. S. Ztschr. physiol. Chem., 185: 245-266. 1929.

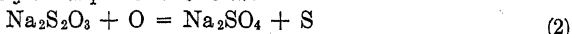
<sup>15</sup> Winogradsky, 1887 (p. 79).

Elementary sulfur was formed as an intermediary product and was actually demonstrated in the cells of the bacteria (*Beggiatoa* and *Thiothrix*). Some organisms like *Thiobacillus thiooxidans* oxidize the  $\text{H}_2\text{S}$  directly to sulfuric acid without forming elementary sulfur, while others, like *Thiobacillus thioparus*, liberate free sulfur outside of their cells. According to Baas-Becking, not the  $\text{H}_2\text{S}$  as such but rather the hydrosulfide ( $\text{HS}^-$ ) is used by the sulfur bacteria as a source of energy.

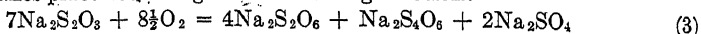
Thiosulfate is oxidized by microorganisms, according to the following group of reactions, depending on the organism taking part in the process:



Free sulfur was found to be liberated in the process. Nathanson<sup>16</sup> considered this to be due to the interaction of the  $\text{Na}_2\text{S}_4\text{O}_6$  and  $\text{Na}_2\text{S}_2\text{O}_3$ . According to Beijerinck,<sup>17</sup> the reaction actually takes place as follows:

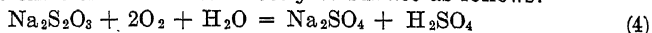


Trautwein<sup>18</sup> suggested that the oxidation of the thiosulfate by the organism that he isolated takes place according to the following reaction:

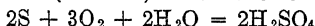


No sulfur was precipitated, the reaction did not become acid.

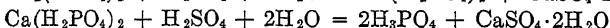
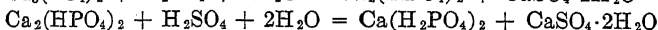
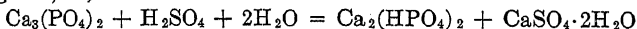
*Th. thiooxidans* oxidizes thiosulfate directly to sulfate as follows:<sup>19</sup>



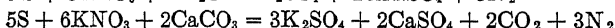
Elementary sulfur is oxidized (table 54) to sulfuric acid:



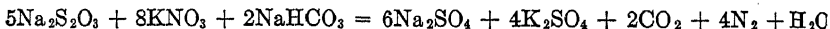
In the presence of tri-calcium phosphate, the sulfuric acid interacts giving first di-calcium phosphate, then mono-calcium phosphate and finally phosphoric acid (figs. 40, 41):



When the sulfur is oxidized by a denitrifying organism, the reaction may take place as follows:



or



The addition of  $\text{CaCO}_3$  frequently stimulates sulfur oxidation in the soil;<sup>20</sup> it also prevents the injurious effect of sulfur oxidation upon the nitrifying bacteria.

Sulfates, even up to 5.0 per cent concentration, do not injure sulfur

<sup>16</sup> Nathanson, 1902 (p. 82).

<sup>17</sup> Beijerinck, 1904 (p. 82).

<sup>18</sup> Trautwein, 1921 (p. 85).

<sup>19</sup> Waksman, S. A. and Starkey, R. L. Jour. Gen. Physiol., 5: 285-310. 1923; Starkey, R. L. Jour. Bact., 10: 135-164, 165-195. 1925.

<sup>20</sup> Brown, H. D. Jour. Amer. Soc. Agron., 15: 350-382. 1923.

oxidation. In this respect the thiosulfate bacteria behave differently from the *Nitrosomonas* and *Nitrobacter*, since the sulfur organisms are not injured by the oxidation products while the nitrite and nitrate bacteria are. The chlorine ion exerts, however, an injurious influence: oxidation of sulfur is reduced by 38 per cent at 0.3 N concentration of NaCl.

The optimum reaction for the activities of *Th. denitrificans* lies at a pH 7.9 to 9.1, with a minimum of pH 3.5. In acid media the reaction

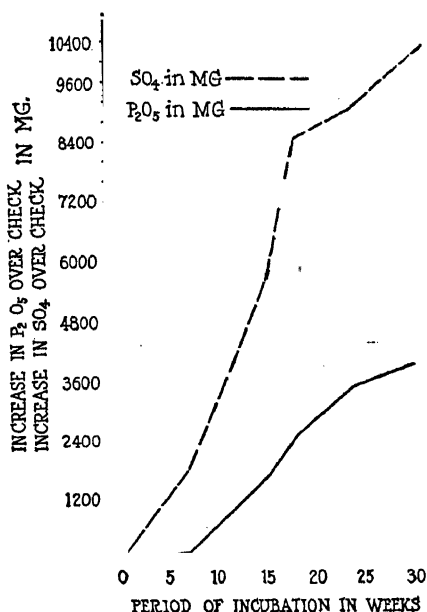


FIG. 40. Course of accumulation of citrate-soluble  $P_2O_5$  and  $SO_4$  in composts of soil, rock phosphate and sulfur (from Lipman, McLean and Lint).

tends to become more alkaline as a result of the activities of the organism, since no free acid is formed.

*Th. thiooxidans* is capable of utilizing elementary sulfur, sulfide and thiosulfate as sources of energy. The carbon is derived only from carbon dioxide. The presence of glucose is not injurious to the growth and sulfur oxidizing capacity of the organism, but it cannot serve either as a source of energy or as a source of carbon. No growth takes place when the cultures are placed in a  $CO_2$ -free atmosphere. Bicarbonates, particularly when the proper reaction of the medium is

obtained by means of an acid, may take the place of  $\text{CO}_2$  only to a small extent, as seen from table 55. The slight increase in acidity in the cultures grown in the  $\text{CO}_2$ -free atmosphere is due to the acidity intro-

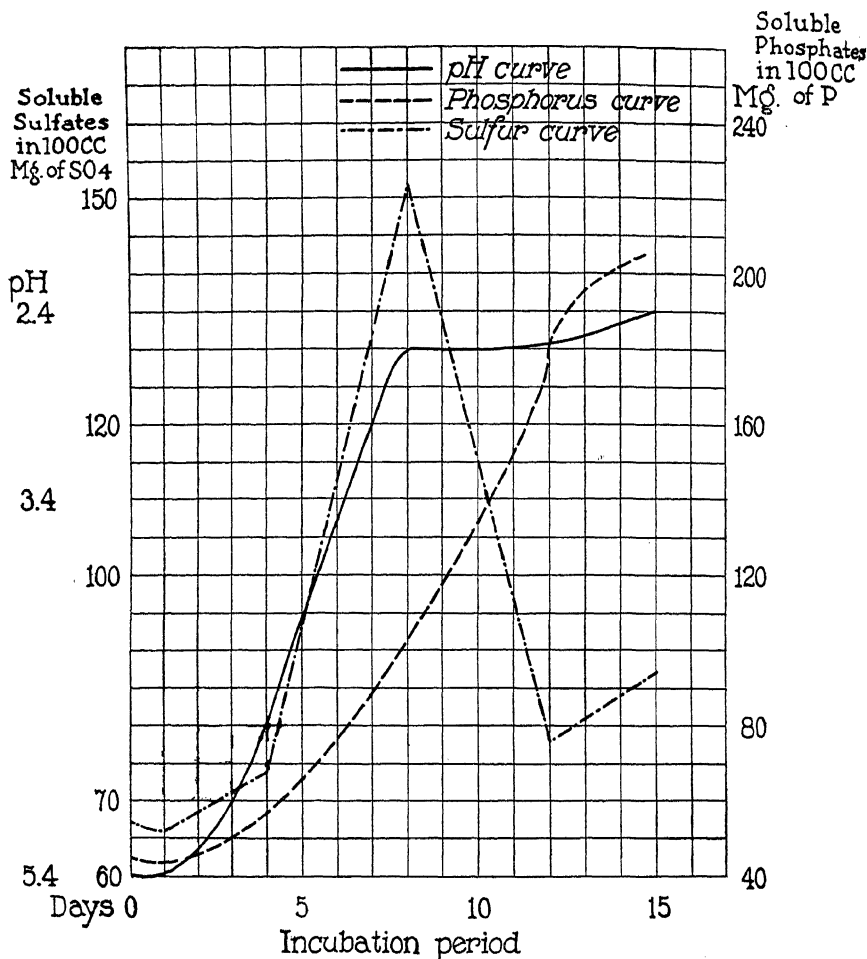


FIG. 41. Course of sulfur oxidation and transformation of insoluble phosphate into soluble forms by *Th. thiooxidans* in liquid media (from Waksman and Joffe).

duced with the inoculum (3 drops per 100 cc. of culture medium). It may be of interest to point out, in this connection, that the optimum reaction for the growth of the organism under laboratory conditions is pH 1.0 to 5.0.

The ratio between the sulfur oxidized and carbon assimilated from  $\text{CO}_2$  of the atmosphere (S:C) was found to be about 32. With thio-sulfate as a source of energy the ratio was found to be about 64. About six and two-thirds per cent of the heat liberated in the oxidation of elementary sulfur is utilized for the chemosynthetic assimilation of carbon. When conditions are unfavorable for the development of the organism, the sulfur-carbon ratio may increase. For example, the presence of nitrates in the medium, which exerts a toxic effect upon the respiration of the organism, resulted in a wider sulfur-carbon ratio. The sulfates were found to have no effect on the respiration of the sulfur oxidizing bacteria, except in very high concentrations. Nitrates are

TABLE 55

*Influence of carbon source upon the growth and sulfur oxidation of Thiobacillus thiooxidans*

TREATMENT	ATMOSPHERE			
	Ordinary		CO <sub>2</sub> -free	
	Final pH	Titre*	Final pH	Titre*
Regular medium, control.....	4.2	2.20	4.2	2.20
Inoculated.....	1.2	12.15	3.8	2.25
1 per cent glucose, control.....	3.0	2.20	3.0	2.2
Inoculated.....	1.2(-)	13.15	2.8	2.3
0.1 per cent $\text{NaHCO}_3$ , control.....	6.6	1.3	6.6	1.3
Inoculated.....	5.4	2.0	6.0	1.75
0.1 per cent $\text{NaHCO}_3 + \text{H}_3\text{PO}_4$ , control.....	6.2	2.2	6.2	2.2
Inoculated.....	1.5	9.3	5.3	2.5

\* Titre = cubic centimeter of 0.1N NaOH necessary to neutralize 10 cc. of medium, with phenolphthalein as indicator.

very toxic, especially in concentrations above 0.2 molar. Phosphates proved to be more toxic than sulfates, but less toxic than nitrates. Peptone becomes toxic in concentrations of 0.05 per cent. The concentration of sulfur in the medium does not exert any important influence on sulfur oxidation; an increase in the quantity of sulfur leads to an increase in oxidation. As to the influence of sulfuric acid or the product of the reaction, it was found that normal growth took place even in concentrations of 0.25 molar, while 0.5 molar did not completely prevent growth.

The influence of sulfates, elementary sulfur, and reaction upon the oxidation of sulfur by *Th. thiooxidans* is shown in fig. 42.

Sulfur may also be oxidized in well aerated soils, rich in organic matter, to soluble and oxidizable sulfur compounds before the sulfates are produced. Hyposulfites form an important group of these intermediary substances. According to Guittonneau,<sup>21</sup> this process is carried out by two different organisms, one of which, an ammonia-forming, hetero-

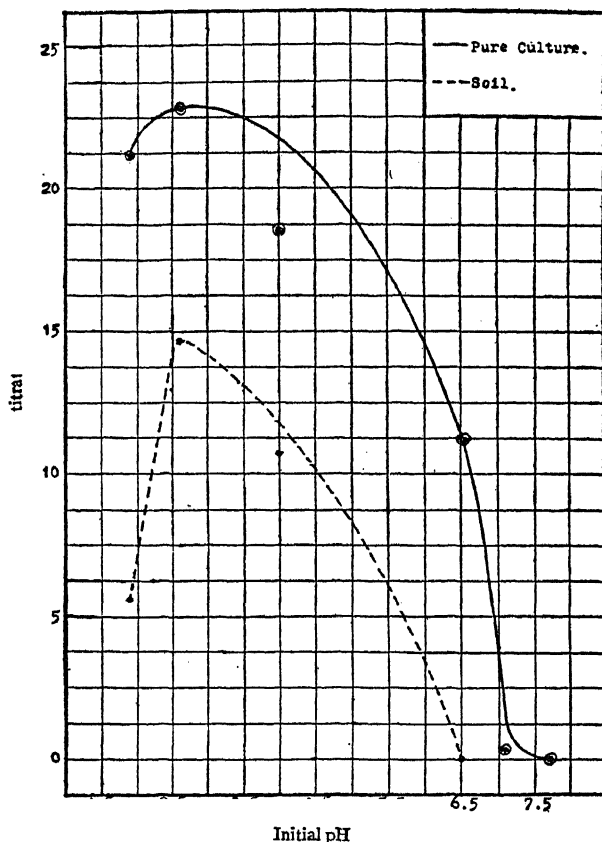


FIG. 42. Influence of hydrogen-ion concentration of medium upon sulfur oxidation by *Th. thiooxidans* (from Waksman and Starkey).

trophic form, oxidizes the sulfur ( $S$ ) to hyposulfite ( $S_2O_2$ ) and the other, a species more or less strictly autotrophic, oxidizes the hyposulfite to

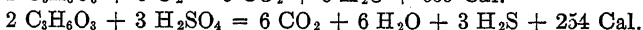
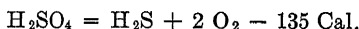
<sup>21</sup> Guittonneau, G. Compt. Rend. Acad. Sci., **180**: 1142-1144. 1925; **181**: 261-262. 1925; **182**: 661-663. 1926; **184**: 898-901. 1927; **191**: 277-278. 1930; First Intern. Congr. Soil Sci., **3**: 274-284. 1928.

sulfate ( $\text{SO}_3$ ). An outside source of energy, either in the form of organic acids, carbohydrates or amino acids, is required. The rapidity of the process depends on the nature of the carbon source and the active organisms. The addition of manure to black-alkali soils favors the rapidity of sulfur oxidation.<sup>22</sup>

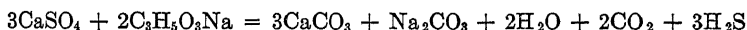
It has been suggested<sup>23</sup> that different sulfides are acted upon by different groups of organisms. Among the iron sulfides, markazite and ferrous sulfide are oxidized more readily than pyrites.

*Reduction of sulfur and its compounds.* When sulfur is added to the soil in an elementary form it is subject to reduction processes, especially when it comes in contact with the living protoplasm of bacteria, fungi, or yeasts; thiosulfates, tetrathionates and pentathionates are also subject to processes of reduction with the formation of  $\text{H}_2\text{S}$ . Rey-Pailhade<sup>24</sup> suggested that this process is enzymatic in nature. When sulfates are added to the soil, they are either assimilated by the plants and microorganisms and transformed into proteins, or are gradually washed out in the drainage waters, or are reduced under anaerobic conditions. This phenomenon was explained<sup>25</sup> as due to the production of nascent hydrogen by the microbes. It was suggested<sup>26</sup> that the oxygen of the sulfate obtained in the reduction process is used for the oxidation of organic matter.

The reduction of sulfate to sulfide requires a large expenditure of energy and accounts for the distinct difference in energy gain when oxidation takes place by means of atmospheric oxygen or when the oxygen is derived from the reduction of sulfates.



The reaction can also be written as follows:



In the presence of cellulose as a source of energy and sulfate as a source of oxygen, methane, acetic and butyric acids are produced from the former and  $\text{H}_2\text{S}$  from the latter.<sup>27</sup> The use of sulfates as a source

<sup>22</sup> Kelley, W. P. and Arany, A. *Hilgardia*, **3**: 393-420. 1928.

<sup>23</sup> Gubin, B. M. *Viestnik Bakt. Agron. Sta.*, **24**: 52-74. 1926.

<sup>24</sup> Rey-Pailhade, J. de. *Compt. Rend. Acad. Sci.*, **118**: 201-203. 1894.

<sup>25</sup> Petri, R. and Maassen, A. *Arb. K. Gesund. Amt.*, **8**: 318, 490. 1893.

<sup>26</sup> Beijerinck and Van Delden, 1904 (p. 155).

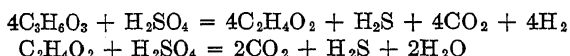
<sup>27</sup> Rubentschik, L. *Centrbl. Bakt. II*, **73**: 483-496. 1928.



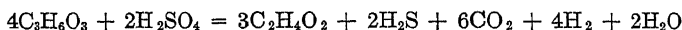
of oxygen is limited to a closely related group of organisms, of which only three strains have been described, including a thermophilic form, *Vibrio thermodesulfuricans*.<sup>28</sup> Both sulfate and thiosulfate can be used as a source of oxygen and salts of organic acids as well as other compounds as sources of carbon.

In crude cultures, the  $H_2S$  formed from the reduction of sulfate, in the presence of organic matter and in contact with oxygen, is again oxidized by the sulfur-oxidizing bacteria. In curative muds and certain lakes there is an increase in the  $H_2S$  content with depth, starting from none at the surface and reaching a concentration of 30 mgm. at a depth of 25 to 30 meters. The hydrogen sulfide formed in the lower layers of the mud is oxidized to sulfate on reaching the surface; the latter is then again reduced when reaching the lower layers.<sup>29</sup>

The presence of sulfur-reducing bacteria in deep layers of earth has been demonstrated by Wolzogen Kühr<sup>30</sup> who found them at depths of 6 to 35 meters. One can readily imagine that processes, similar to those taking place in muds, may also occur in the soil. The requirements for sulfate-reduction by *Microspira desulfuricans* are (1) absence of free oxygen, (2) presence of organic compounds as sources of energy, (3) presence of sulfate as a source of oxygen, and (4) presence of essential inorganic elements in available forms. The following reactions will then take place:



or



Certain actinomyces are also capable of reducing sulfates to hydrogen sulfide.<sup>31</sup>

*Formation of  $H_2S$  in the decomposition of organic matter.* It has been pointed out elsewhere that a large number of bacteria, including aerobic and anaerobic forms, are capable of forming hydrogen sulfide and other volatile sulfur compounds in the decomposition of organic matter

<sup>28</sup> Elion, L. Centrbl. Bakt. II, **63**: 58-67. 1924.

<sup>29</sup> A detailed review of this subject is given by Nadson, G. On the hydrogen sulfide fermentation in the Veissovo-Salt lake and the part played by the micro-organisms in the formation of black mud. St. Petersburg 1903; Dügge, 1919 (p. 80).

<sup>30</sup> von Wolzogen Kühr, C. A. H. Proc. Roy. Soc. Amsterdam, **25**: 188-198, 1922. (Chem. Abstr., 17: 47.)

<sup>31</sup> Sawjalow, W. Centrbl. Bakt. II, **39**: 440-447. 1913.

containing sulfur. Proteins may contain as much as 1.5 per cent of sulfur; on hydrolysis, the sulfur is liberated from them partly in the form of  $H_2S$ . This process is carried out by a large number of bacteria, especially certain obligate (*Bac. putrificus*, *Bac. sporogenes*) and facultative (*Bact. coli*, *Bact. vulgare*, *Staph. pyogenes aureus*) anaerobes.

The formation of  $H_2S$  can be tested by the use of a piece of bibulous paper saturated with lead acetate to which a little glycerol has been added. The paper is suspended in the upper part of the container over the growing culture; it will at first become brown, then black. To determine the numbers of organisms in the soil capable of forming  $H_2S$  from a specific organic material, it is sufficient to incorporate some lead acetate into an agar medium; the formation of a dark color will indicate the ability of the organism to produce  $H_2S$ .<sup>32</sup>

The differences in the amounts of  $H_2S$  formed by various organisms are quantitative rather than qualitative in nature;<sup>33</sup> they are largely determined by the nature of the medium and oxygen tension. According to Rubner,<sup>34</sup> *Bact. vulgare* produced 33 mgm. of  $H_2S$  under anaerobic conditions, and only 4 to 5 mgm. under aerobic conditions in one liter of peptone-free bouillon. Quantitatively, the  $H_2S$  is determined by the difference in the total sulfur content of the medium.<sup>35</sup> The gases formed may be absorbed in standard iodine solution from which the sulfide is determined by titration with thiosulfate. When organic sulfur compounds are decomposed by the agency of micro-organisms, most of the sulfur may be assimilated and resynthesized, as shown by Rubner for *Bact. vulgare* grown in a liter of bouillon:

	BEFORE THE EXPERIMENT	AT THE END OF THE EXPERIMENT	DIFFERENCE
	mgm.	mgm.	mgm.
Sulfate S.....	6.1	1.5	-4.6
Organic S.....	52.8	28.1	-24.7
Iron precipitated S, in the bacterial cells..	1.2	25.3	+24.1

The loss of 5.2 mgm. is due to the formation of  $H_2S$ , while the larger portion of the organic sulfur is transformed into microbial protoplasm.

<sup>32</sup> Burnet, E. and Weissenbach, R. J. Compt. Rend. Soc. Biol., 78: 565-568. 1915.

<sup>33</sup> A detailed study of the organisms concerned in the formation of  $H_2S$  from various sulfur compounds is recorded by Sasaki, T. and Otsuka, I. Biochem. Ztschr., 39: 208-215. 1912; Myers, J. T. Jour. Bact., 5: 231-252. 1920.

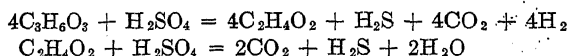
<sup>34</sup> Rubner, M. Arch. Hyg. 16: 78-100. 1893.

<sup>35</sup> See Almy, L. H. Jour. Amer. Chem. Soc., 47: 1381-1390. 1925.

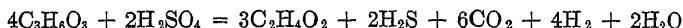
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<sup>31</sup> Sawjalow, W. Centrbl. Bakt. II, **39**: 440-447. 1913.

containing sulfur. Proteins may contain as much as 1.5 per cent of sulfur; on hydrolysis, the sulfur is liberated from them partly in the form of  $H_2S$ . This process is carried out by a large number of bacteria, especially certain obligate (*Bac. putrificus*, *Bac. sporogenes*) and facultative (*Bact. coli*, *Bact. vulgare*, *Staph. pyogenes aureus*) anaerobes.

The formation of  $H_2S$  can be tested by the use of a piece of bibulous paper saturated with lead acetate to which a little glycerol has been added. The paper is suspended in the upper part of the container over the growing culture; it will at first become brown, then black. To determine the numbers of organisms in the soil capable of forming  $H_2S$  from a specific organic material, it is sufficient to incorporate some lead acetate into an agar medium; the formation of a dark color will indicate the ability of the organism to produce  $H_2S$ .<sup>32</sup>

The differences in the amounts of  $H_2S$  formed by various organisms are quantitative rather than qualitative in nature;<sup>33</sup> they are largely determined by the nature of the medium and oxygen tension. According to Rubner,<sup>34</sup> *Bact. vulgare* produced 33 mgm. of  $H_2S$  under anaerobic conditions, and only 4 to 5 mgm. under aerobic conditions in one liter of peptone-free bouillon. Quantitatively, the  $H_2S$  is determined by the difference in the total sulfur content of the medium.<sup>35</sup> The gases formed may be absorbed in standard iodine solution from which the sulfide is determined by titration with thiosulfate. When organic sulfur compounds are decomposed by the agency of micro-organisms, most of the sulfur may be assimilated and resynthesized, as shown by Rubner for *Bact. vulgare* grown in a liter of bouillon:

	BEFORE THE EXPERIMENT	AT THE END OF THE EXPERIMENT	DIFFERENCE
	mgm.	mgm.	mgm.
Sulfate S.....	6.1	1.5	-4.6
Organic S.....	52.8	28.1	-24.7
Iron precipitated S, in the bacterial cells..	1.2	25.3	+24.1

The loss of 5.2 mgm. is due to the formation of  $H_2S$ , while the larger portion of the organic sulfur is transformed into microbial protoplasm.

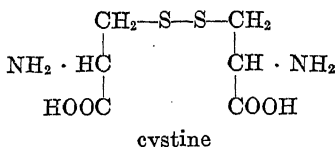
<sup>32</sup> Burnet, E. and Weissenbach, R. J. Compt. Rend. Soc. Biol., 78: 565-568. 1915.

<sup>33</sup> A detailed study of the organisms concerned in the formation of  $H_2S$  from various sulfur compounds is recorded by Sasaki, T. and Otsuka, I. Biochem. Ztschr., 39: 208-215. 1912; Myers, J. T. Jour. Bact., 5: 231-252. 1920.

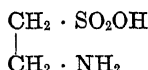
<sup>34</sup> Rubner, M. Arch. Hyg. 16: 78-100. 1893.

<sup>35</sup> See Almy, L. H. Jour. Amer. Chem. Soc., 47: 1381-1390. 1925.

When proteins and other sulfur bearing organic materials are added to the soil they are hydrolyzed by the activities of microorganisms into the various constituent groups and the sulfur-bearing amino acid, cystine or di-cystine, or di- $\beta$ -thio- $\alpha$ -amino-propionic acid is liberated.



Another source of sulfur in an organic form is bile which contains taurine.



These substances are decomposed in the soil or in culture media by microorganisms. The sulfur is liberated as  $\text{H}_2\text{S}$  or in the form of mercaptans,<sup>36</sup> depending on the organisms and environmental conditions, especially oxygen supply. The production of mercaptan from *l*-cystine by *Bact. vulgare* is not affected by the presence of glucose, lactose and glycerol. *Bact. vulgare* and *B. coli* are also capable of forming  $\text{H}_2\text{S}$  and ethyl sulfide from *l*-cystine, independent of the presence of the carbon sources just named. Mercaptans, either the ethyl or methyl form ( $\text{C}_2\text{H}_5 \cdot \text{HS}$  or  $\text{CH}_3 \cdot \text{HS}$ ), often accompany  $\text{H}_2\text{S}$  as decomposition products of proteins under anaerobic conditions. These are often produced in mere traces. A definite parallelism has been found between the influence of carbohydrates on bacterial multiplication and on the production of  $\text{H}_2\text{S}$  from proteins. The rate of formation of  $\text{H}_2\text{S}$  even increases in the presence of glucose, although the formation of amino-nitrogen remains stationary.<sup>37</sup>

Taurine is very resistant to the action of microorganisms.<sup>38</sup> According to Rippel,<sup>39</sup> cystine and thiourea are decomposed in the soil by fungi, resulting in the oxidation of the sulfur to sulfate.

*Influence of sulfur oxidation upon the transformation of minerals in the soil.* The acid produced from the oxidation of elementary sulfur

<sup>36</sup> Petri and Maassen, 1893 (p. 535); Kondo, M. *Biochem. Ztschr.*, **136**: 198-202. 1922.

<sup>37</sup> Heap, H. and Cadness, B. H. E. *Jour. Hyg.*, **23**: 77-93. 1924.

<sup>38</sup> Sasaki and Otsuka, 1912 (p. 537).

<sup>39</sup> Rippel, A. *Biochem. Ztschr.*, **165**: 473-474. 1925.

by microorganisms can be utilized (1) as a solvent for such difficultly soluble minerals, as phosphorus in rock phosphate, potassium in glauconite, or greensand marl; (2) for the neutralization of excess base in alkaline soils; (3) for the control of certain plant diseases.

When a compost of soil, sulfur and insoluble calcium phosphate is made, the sulfuric acid formed from the oxidation of the sulfur acts upon the phosphate and makes it soluble. By composting 100 grams of soil, 5 grams of flowers of sulfur and 15 grams ground rock phosphate, 85 per cent of the phosphate is made available after a period of 30 weeks.<sup>40</sup> A compost consisting of 100 parts of soil, 120 parts of sulfur

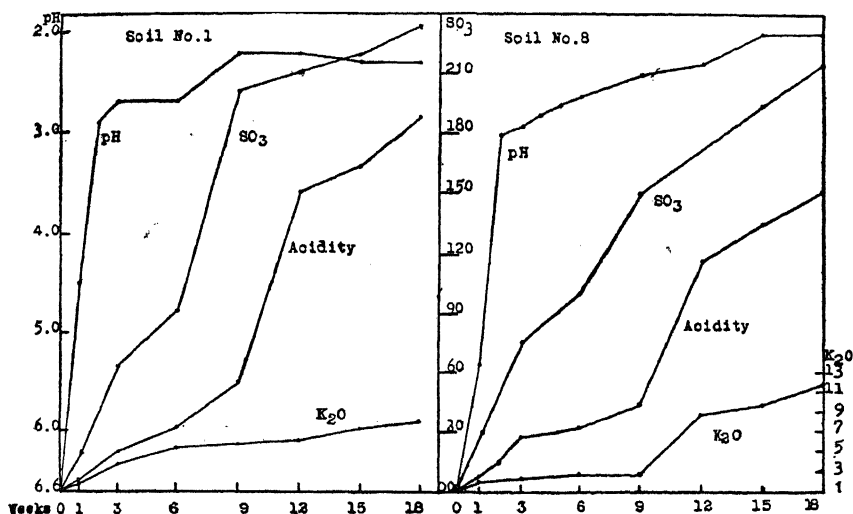


FIG. 43. Relation between sulfur oxidation and water-soluble potassium in composts containing sulfur and greensand marl (from Rudolfs).

and 400 parts of rock phosphate is the most economical combination for the production of phosphoric acid. For the maximum transformation of the phosphate, a compost of equal amounts of sulfur, phosphate and a large amount of soil is required. When a fresh compost is inoculated with some material from an old compost, the reaction takes place much more rapidly. Abundant aeration and optimum moisture offer favorable conditions; small amounts of ferrous and aluminum sulfates exert a stimulating effect. Low grade phosphorite can thus be made an available source of phosphorus for plant growth, by compositing it with

<sup>40</sup> Lipman, J. G., McLean, H. C. and Lint, H. C. *Soil Sci.*, 2: 499-538. 1916; 5: 251-290. 1918.

sulfur.<sup>41</sup> The uncomposted mixture cannot be added as such to the soil since the phosphate will not become soluble under soil conditions. The reaction of the soil would have to be made highly acid, before the transformation would take place.

The same reactions take place in sterile liquid media inoculated with a pure culture of *Th. thiooxidans*. By adding 1 gram of powdered sulfur and one gram of chemically pure tri-calcium phosphate to 100 cc. of a synthetic solution, sterilizing and inoculating with a pure culture of the organism, certain curves are obtained (fig. 41).<sup>42</sup>

Composts prepared from greensand and sulfur allow the liberation of small amounts of potassium at pH 2.7 to 2.3 (fig. 43); this potassium when introduced into the soil is readily assimilated by plants.<sup>43</sup>

Another interesting process in which oxidation of sulfur may be utilized is the reduction of the alkalinity of black alkali soils. In view of the fact that the high alkalinity of such soils is due not only to the presence of sodium carbonate but to the fact that sodium forms the saturating base in the zeolitic silicates, large quantities of sulfur have to be applied before any marked effect is observed. The sulfur is readily oxidized and the sulfuric acid changes the carbonates to bicarbonates and then to sulfates. However, when insufficient amounts of sulfur are applied, the zeolitic sodium will soon form fresh carbonates and the reaction of the soil again becomes markedly alkaline. The acid not only neutralizes the carbonates, but coagulates the colloids, thus making the soil more permeable, allowing leaching to take place.<sup>44</sup>

An increase in the acidity of the soil which results from the application of sulfur can be utilized for the control of organisms which cause plant diseases, such as *Act. scabiei* causing potato scab. However, an increased acidity may prove injurious to microorganisms whose activities are essential for the normal biochemical soil processes, such as the bacteria concerned in the process of nitrification, etc.<sup>45</sup> The practical application of sulfur must, therefore, always be accompanied by a careful study of the reaction of the soil and the various chemical and biological changes which may result from increased acidity.

<sup>41</sup> Kalushsky, A. Ztschr. Pflanz. Düng. Bodenk A, **12**: 217-226. 1928.

<sup>42</sup> Waksman, S. A. and Joffe, J. S. Jour. Biol. Chem., **50**: 35-45. 1922.

<sup>43</sup> Rudolfs, W. Soil Sci., **14**: 307-319. 1922.

<sup>44</sup> This process was studied in detail by Hibbard, P. L. Soil Sci., **11**: 385-387. 1921; Rudolfs, W. Soil Sci., **13**: 215-229. 1922; Joffe, J. S. and McLean, H. C. Soil Sci., **17**: 395-409. 1924; **18**: 13-30, 133-149, 237-251. 1924.

<sup>45</sup> The influence of sulfur oxidation upon microbiological activities in the soil is discussed in detail by Boulanger and Dujardin. Compt. Rend. Acad. Sci., **155**: 327. 1912; Vogel, J. Centrbl. Bakt. II, **40**: 60-83. 1914.

PART D

SOIL MICROBIOLOGICAL PROCESSES AND SOIL FERTILITY

*. . . . la terre végétale est regardée comme un support  
actif, une chose vivante . . . .—M. BERTHELOT*





## CHAPTER XXII

### THE SOIL AS A MEDIUM FOR THE GROWTH AND ACTIVITIES OF MICROORGANISMS

The chemical condition of the entire crust of the earth may be considered to be a result of the activities of living organisms.<sup>1</sup> Although the energy which characterizes the biosphere is entirely of cosmic origin, it is changed into chemical soil complexes through the agency of living organisms. These, by their processes of growth, reproduction and nutrition, death and decomposition, as well as the production of numerous forms of life through countless generations have made the surface of the earth what it is to-day, namely a soil teeming with life and supporting within it and upon it the animal, plant and microbiological populations. The microorganisms play a highly important part in the life processes taking place in the soil and contribute materially to the growth of higher plants and animals deriving directly or indirectly their energy from the soil. To be able to understand these processes, we must understand first the nature of the medium, the soil, in which these organisms live, act and reproduce.

*The soil as a culture medium.* The soil is a complex system consisting of (a) mineral particles formed as a result of mechanical and chemical decomposition of the various mineral constituents of native rocks; (b) organic matter of plant, animal and microbial origin, in the process of decomposition or resisting further decomposition; (c) compounds formed as a result of interaction of substances produced in the decomposition of organic matter with materials resulting from the disintegration of the inorganic soil complexes; (d) soil moisture, containing in solution  $\text{CO}_2$ , oxygen and other gases and various organic compounds and inorganic salts; and finally (e) soil atmosphere.

The soil is regarded<sup>2</sup> as a mineral framework, the particles of which are coated with a jelly-like layer of organic and inorganic materials

<sup>1</sup> Vernadsky, W. Centrbl. Mineral, B, No. 11: 583-594. 1928.

<sup>2</sup> Page, H. J. Trans. Faraday Soc., 17: 272-287. 1922. According to Bouyoucos, colloids do not exist in the soil entirely as a coating around the soil grains, but also as independent components; sand particles may not be covered with colloidal gel (Soil Sci., 21: 481-488. 1926.)

present in a colloidal condition, the soil solution being present partly in the colloidal layer and partly in a free condition. The microbial population of the soil lives largely in the colloidal layer and partly in the soil solution. The activities of the microorganisms living in the soil as a medium are affected by the nature of the different ingredients of the medium and by the various conditions influencing them.

The complex medium supplies to the various organisms inhabiting it the necessary nutrients, of an organic and inorganic nature, consisting of the elements oxygen, hydrogen, carbon, nitrogen, phosphorus, sulfur, potassium, magnesium, calcium and iron; and, to a less extent, chlorine, silicon, sodium, aluminum and manganese. The availability of these nutrients as well as their utilization will be greatly influenced not only by the (1) physical and chemical composition of the solid part of the soil, but also by the (2) composition of the soil atmosphere, (3) composition of the soil solution, (4) reaction of the soil and (5) soil temperature. When any one of these factors is changed there is a correlated change in the biological composition of the soil; i.e., any modification in the physical, chemical or physico-chemical conditions of the soil will greatly affect the biological flora and its activities.<sup>3</sup> We possess only fragmentary information concerning the biological responses to modifications of these soil conditions.

*Soil composition and microbiological activities.* The solid, liquid, and gaseous phases of the soil influence individually and collectively the distribution and activities of microorganisms. The mineral framework is of prime importance. It is made up of mineral matter derived from the disintegration of rock materials, and of calcium carbonate, calcium phosphate and organic matter deposited in the soil by the various marine organisms during soil formation. The organic soil constituents contributing to the soil as a medium for the growth of microorganisms are (1) undecomposed plant and animal residues of recent origin; (2) the intermediary substances, the more or less inert constituents of the original organic matter added to the soil, and the products of decomposition of plant and animal substances; finally (3) the various dead and living cells of microorganisms and the products resulting from their decomposition.

The mineral framework influences the activities of microorganisms by modifying the mechanical condition of the medium, by forming substances, such as the zeolitic silicates, which, either themselves or by

<sup>3</sup> Lantzsch, K. *Centrbl. Bakt.* II, 54: 1-12. 1921; König, J. and Hasenbäumer, J. *Landw. Jahrb.*, 55: 185-252. 1920.

interaction with certain of the organic compounds, form the colloidal matter in which most of the microbial transformations take place, by offering direct mineral nutrients to the microorganisms, and by combining with various products of the metabolism of microorganisms, such as the organic and inorganic acids.

The inorganic as well as the organic soil colloids give to the soil such desirable properties as its capacity for absorption and retention of water and bases, and its buffering action, which regulates changes in reaction. They may also exert a direct influence upon the distribution and nature of action of microorganisms in the soil.

The organic matter of the soil gives to it a brown to black color. The higher moisture holding capacity of the soil is due to a large extent to a higher content of organic matter. Carbon is present in the soil chiefly in the various organic substances collectively termed humus and in the form of carbonates. The nitrogen is present in the soil in the form of complex proteins and their derivatives,<sup>4</sup> as well as other complex nitrogenous compounds largely derived from the cells of microorganisms inhabiting the soil; only about one per cent of the nitrogen of the soil is present there as ammonia and nitrates. The various minerals required by the microorganisms for their activities are present partly in the mineral framework, partly in the organic and inorganic soil colloids, partly in the soil solution, and partly in a precipitated form.

The amount of moisture<sup>4</sup> that a soil can hold varies with the size of its particles; it will be low in the coarse sandy soils and greater in the fine clay and especially in the peat soils, which consist largely of organic matter in a colloidal state.<sup>5</sup> The minimum and optimum amounts of moisture for the activities of microorganisms depend upon the nature of the soil and its colloidal content. Using the evolution of CO<sub>2</sub> from glucose as an index of the activities of microorganisms, Van Suchtelen<sup>6</sup> found that when a loam soil contained only 4.4 per cent moisture, the activities were at a standstill; when the moisture was increased to 6 per cent, 19 mgm. of CO<sub>2</sub> were formed, and with 15 per cent moisture, 208 mgm. were formed. Similar results were obtained when the number

<sup>4</sup> Jodidi, S. L. Iowa Agr. Exp. Sta. Res. Bul. 1. 1911; Potter, R. S. and Snyder, R. S. Jour. Agr. Res., 6: 61. 1916.

<sup>5</sup> Soil sampling as well as moisture determination is discussed in detail by Heine, 1928 (p. XVI) and Stoklasa, Abderhald. Handb. biochem. Arbeitsmeth. 5, Pt. 2, 843-910. 1925.

<sup>6</sup> Van Suchtelen, 1910 (p. 609); Engberding, 1909 (p. 14).

of bacteria developing on the plate was used as an index of microbiological activities. Optimum moisture conditions, for the activities of many soil microorganisms, are reached when about half the pore space of the soil is filled with water. In the case of light sandy soils this condition obtains when the water content is about 8 to 10 per cent of its weight and, in heavy silt or clay soils, when the water content is 16 to 20 per cent or more. As the soil dries out the activities of the organ-

TABLE 56

*Influence of moisture upon the formation of ammonia and nitrate (from horn meal) in different soils*

SOIL TYPE	MOISTURE CONTENT	PERIOD OF INCUBATION	AMMONIA AND NITRATE NITROGEN
	<i>per cent</i>	<i>weeks</i>	<i>per cent</i>
Sand.....	6	3	45.7
		12	60.2
	12	3	42.4
		12	55.5
	18	3	38.5
		12	55.0
Loam.....	8	3	18.9
		12	49.7
	16	3	45.5
		12	53.9
	24	3	39.5
		12	39.0
Clay.....	8	3	0.6
		12	15.4
	18	3	29.1
		12	54.9
	28	3	36.9
		12	24.2

isms are gradually reduced; the larger forms, like the fungi, suffer most while the smaller and more resistant forms, like the actinomyces and spores of bacteria,<sup>7</sup> suffer least. Excessive moisture may prove unfavorable to aerobic microorganisms by limiting the supply of oxygen, while anaerobic bacteria are favored.

Since the microorganisms live largely upon the colloidal film surrounding the inorganic soil particles, the lower the colloidal content of a

<sup>7</sup> Hoffmann, C. Wis. Agr. Exp. Sta. Res. Bul. 23. 1912.

soil the smaller is the amount of water necessary to bring it into a condition favorable for the activities of microorganisms. An excess above the optimum amount of moisture will prove injurious to the activities of the aerobic organisms. The heavier the soil, the higher must be the water content to bring about equal decomposition of organic nitrogenous substances. Using the liberation of ammonia and nitrate from horn meal as an index of the activities of microorganisms, Münter<sup>8</sup> demonstrated (table 56) that in a sandy soil decomposition did not vary within the moisture range of 6 to 18 per cent (decreasing at greater moisture contents). The rapidity of decomposition reached its highest point during the first three weeks in the sandy soil. In a loam soil, decomposition was twice as great at 16 per cent moisture as at 8 per cent, and was constant between 16 and 24 per cent. The influence of the moisture content was still more marked in a clay soil. Only 0.63 per cent of the material was decomposed in three weeks at 8 per cent moisture, while at 18 and 28 per cent moisture and in the same period of time, 30.80 and 36.36 per cent of the material was decomposed respectively. After twelve weeks, 13.35, 63.21 and 57.94 per cent of the material was decomposed at the respective moisture contents.

The effect of moisture upon the composition of the soil flora and fauna is very marked; the nature of the processes occurring in the soil will consequently be materially affected. The decomposition of cellulose can readily illustrate this phenomenon. In the presence of sufficient available nitrogen and minerals, cellulose is decomposed at a medium moisture content by filamentous fungi and aerobic bacteria; at a low moisture content, by filamentous fungi and actinomyces; when the soil is saturated with water, anaerobic bacteria are largely concerned with the decomposition of the cellulose. When sugar is added to the soil and the development of microorganisms is followed by direct microscopic examination, *Azotobacter* is found to develop under aerobic and *Cl. pastorianum* under anaerobic conditions, which may be reached below the surface even when the moisture content of the soil is only 40 per cent of saturation.<sup>9</sup>

The influence of moisture upon the activities of bacteria is due to two factors:<sup>10</sup> (1) penetration of atmospheric oxygen through the medium;

<sup>8</sup> Münter, F. Landw. Jahrb., 55: 62-138. 1920.

<sup>9</sup> Winogradsky, 1924 (p. 475).

<sup>10</sup> Rahn, O. Centrbl. Bakt. II, 38: 484-494. 1913; Mich. Agr. Exp. Sta. Tech. Bul. 16. 1912.

(2) rapidity of diffusion of nutrients. A high moisture content is more favorable for bacteria, but it diminishes soil aeration; when the soil is saturated with water, oxygen can penetrate only by solution, which is too slow for the development of aerobic organisms. When the moisture content of the soil is diminished, aeration increases. The larger the inorganic soil particles the greater are the possibilities for aeration. The smaller the inorganic soil particles, the greater is the amount of moisture required for the activities of microorganisms and the less is the soil aerated.

Aeration of the soil or the diffusion of the soil gases greatly influence the activities of microorganisms. Oxygen is needed for oxidation processes,  $\text{CO}_2$  for the activities of autotrophic bacteria, and nitrogen for the nitrogen-fixing organisms.<sup>11</sup> Oxidation favors the activities of nitrate-forming and nitrogen-fixing bacteria, fungi, actinomyces, and other organisms which bring about the decomposition of organic matter. The processes of decomposition may be so rapid in well aerated soils that farmers must resort to rolling and manuring in order to maintain the proper supply of organic matter in the soil. In heavy fine-grained soils with insufficient aeration, the decomposition of organic matter may be too slow and the farmer may use drainage, tillage, liming and manuring to intensify aeration. Insufficient aeration favors reduction processes.

*The mineral composition of the soil.* While nitrogen and, to a large extent, sulfur are present in the soil almost entirely in organic forms and the carbon utilized by the heterotrophic organisms is also of complex organic nature, the other elements are largely or entirely of inorganic origin. Phosphorus exists in the soil as apatite ( $\text{Ca}_5(\text{PO}_4)_3\cdot\text{Cl}$  or  $\text{Ca}_5(\text{PO}_4)_3\cdot\text{F}$ ), calcium phosphate ( $\text{Ca}_3(\text{PO}_4)_2$ ), iron and aluminum phosphates, as well as in various organic combinations. Potassium occurs in the soil in orthoclase and microcline feldspar ( $\text{KAlSi}_3\text{O}_8$ ), in muscovite mica ( $\text{KH}_2\text{Al}_3\text{Si}_3\text{O}_{12}$ ) and in hydrated and non-hydrated aluminum silicates and to a less extent in organic combination. Calcium exists in the soil in various minerals such as calcite, plagioclase, feldspar, hornblende and augite, and in absorbed compounds with kaolinite, etc.

Table 57 shows the composition of some typical American soils compared with the composition of the lithosphere.<sup>12</sup> Practically every soil

<sup>11</sup> Russell, E. J. and Appleyard, A. Jour. Agr. Sci., 7: 1-48. 1915; 8: 385-417. 1917.

<sup>12</sup> Robinson, W. O. U. S. Dept. Agr. Bur. Soils, Bul. 122. 1915. Methods of chemical and mechanical soil analysis are discussed by Russell and others (see list of texts).

contains most of the rock-forming minerals; these are found both in the sand and in the clay fractions of the soil.<sup>13</sup>

It is only seldom that the mineral constituents become limiting factors to the development of microorganisms in normal soils. These minerals influence the activities of the organisms by modifying the soil reaction, the concentration of the soil solution, by serving as direct nutrients and in some cases even as sources of energy.

The nature and composition of the various horizons, however, have an important influence upon the distribution of microorganisms in the soil.

TABLE 57

*Comparative mineral composition of surface soil and of lithosphere*

COMPOUNDS	AMERICAN SURFACE SOILS	LITHOSPHERE
	<i>per cent</i>	<i>per cent</i>
SiO <sub>2</sub> .....	84.67	59.77
Al <sub>2</sub> O <sub>3</sub> .....	6.73	14.89
TiO <sub>2</sub> .....	0.66	0.77
Fe <sub>2</sub> O <sub>3</sub> .....	2.53	6.25
MnO.....	0.06	0.09
Na <sub>2</sub> O.....	0.49	3.25
K <sub>2</sub> O.....	1.03	2.98
CaO.....	0.40	4.86
MgO.....	0.27	3.74
P <sub>2</sub> O <sub>5</sub> .....	0.09	0.28
SO <sub>3</sub> .....	0.09	0.28
N <sub>2</sub> .....	0.07	....
Organic matter.....	2.61	....
Carbon.....	1.51	0.03

A typical podsol soil profile is shown in fig. 44.<sup>14</sup> According to Weis,<sup>15</sup> the true colloidal organic complexes representing the highest nitrogen content have, of all the soil organic material, the greatest power of moving into the lower layers, so that the nitrogen content increases with increasing depth. The limited information concerning the distribution of microorganisms in the various soil horizons<sup>15a</sup> indicates

<sup>13</sup> McCaughey, W. J. and Fry, W. H. U. S. Dept. Agr. Bur. Soils, Bul. 91. 1913; Hart, R. Jour. Agr. Sci., 19: 90-105. 1929; Merrill, (p. XVI); Dana, E. S. Text-book of mineralogy. 3d Ed., J. Wiley. 1922.

<sup>14</sup> Müller, P. E. 1878 (p. 676).

<sup>15</sup> Weis, Fr. Det. Kgl. Danske Videnskab. Selsk. Biol. Meddel. VII, 9. 1929.

<sup>15a</sup> Brown, P. E. and Benton, T. H. Iowa Agr. Exp. Sta., Res. Bul., 132: 1930; Cononova, M. M. Contrib. Central Asiatic Inst., 7: 53-67. 1930.



that the chemical and physical soil conditions have a marked influence upon the nature and distribution of microorganisms. The largest numbers are found in the A horizons, with a rapid decrease in the B horizon and a practical disappearance of most microorganisms in the C horizon.

*The physico-chemical rôle of organic matter in the soil.* Organic matter plays a manifold rôle in the soil. Most microorganisms find in it their source of energy and other nutrients. Its influence on soil texture is of

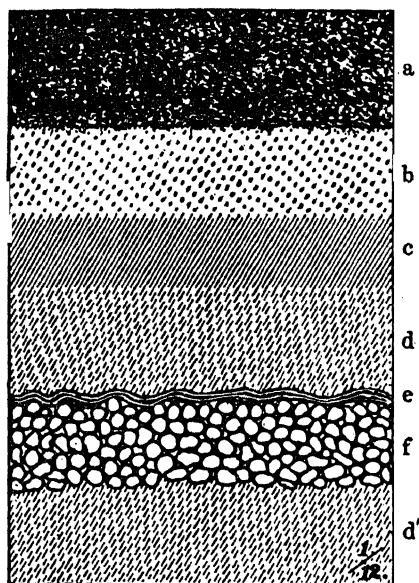


FIG. 44. Forest soil profile: a. organic horizon, b. grayish white clay, c. dark brown humus-rich clay, d. and d' blue-gray plastic clay, e. irregular compact layer rich in iron, f. layer of pebbles (from P. E. Müller).

especial importance. Organic matter helps to loosen a clay soil and add body to a sandy soil. It is best to apply only undecomposed organic matter to heavy soils, since the large quantities of  $\text{CO}_2$  produced in the process of decomposition tend to make the heavy soil porous. It is best to subject the organic matter first to partial decomposition before applying it to sandy soils, so as not to make the soil too open. The colloidal humus seems to have a cementing effect upon the coarser soil particles. It also exerts a protective effect upon clay, so that greater concentrations of electrolytes are required for its flocculation.

Due to its large capacity for absorbing water, the soil organic matter causes a swelling of the soil when wetted. Successive drying, moistening and freezing modify the nature of the organic matter and make it more available to the activities of microorganisms with the production of larger amounts of  $\text{CO}_2$  and nitrate. When a soil is well cultivated, a loose state of aggregation results which allows a rapid development of fungi, actinomyces and aerobic heterotrophic bacteria. These decompose the organic matter, use the available oxygen, and form  $\text{CO}_2$  as one of the chief products. The gases bring about a further extension of the spaces between the soil particles and create a condition referred to in German as "Bodengare." An improved tilth results. This condition is favored by fertilizing the soil with stable or green manure, by liming it and by thorough cultivation, under proper moisture conditions. The colloidal organic matter increases the water holding capacity of the soil and helps to warm it more rapidly, due to a decrease in the evaporation of water, to better drainage and to absorption of the radiant heat of the sun. The organic colloids exert a definite buffer effect upon the reaction of the soil solution;<sup>16</sup> they also regulate the composition and concentration of the solution.

*Colloidal condition of soils and microbiological activities.* Colloids are characterized by a group of reactions which were believed originally to differentiate them sharply from crystalloids, and especially by the fact that they show no marked tendency to diffuse in solutions or to pass through semi-permeable membranes. With the advance of our knowledge of colloids, it has been found that no sharp line of demarcation can be drawn between them and crystalloids. Both conditions depend on the method of preparation, including the nature of the solvent employed. We speak now of colloidal systems rather than of colloidal substances. Colloidal systems are not stable. Colloids are also characterized by extended surfaces which allow them to absorb water and dissolved substances from solution. The attraction for water is both chemical and physical. Heat of wetting is characteristic of soil colloids. An active colloid may be changed to an inactive one by changing its physical condition (ignition) and also to some extent by changing its chemical condition.<sup>17</sup>

Soil colloids consist largely of silica, alumina, iron oxides and organic matter.<sup>18</sup> The plant and animal residues added to the soil are

<sup>16</sup> Oden, S. Trans. Faraday Soc., 17: 288-294. 1922.

<sup>17</sup> Anderson, M. S. Jour. Agr. Res., 28: 927-936. 1924; Bouyoucos, G. J. Soil Sci., 19: 477-483. 1925.

<sup>18</sup> Robinson, W. O. and Holmes, R. S. U. S. Dept. Agr. Bul. 1311. 1924.

largely complexes (proteins, celluloses, starches, lignins) in a colloidal state. When these complexes are acted upon by microorganisms they are partly transformed into crystalloids, and partly into new colloids, namely the soil organic matter or humus. The clay portion of the soil is colloidal in nature.

Colloids are usually separated from crystalloids by dialysis, ultra-filtration, and centrifuging. The osmotic pressure of colloidal solution is small, due to the large molecular weight; diffusion is, therefore, also small; the lowering of the freezing point is small. When colloids are transformed by microorganisms into crystalloids, the molecular weight is decreased with a corresponding increase in osmotic pressure, diffusion and lowering of freezing point. A study of the chemical properties of the proteins as organic colloids led to the conclusion<sup>19</sup> that the theory of amphoteric colloids is in its general features identical with the theory of inorganic metal hydroxides.

The colloidal properties of soil which are of special importance in the growth of microorganisms in soil as a culture medium are (1) the absorption of substances from solution and their concentration upon the surface of the colloid, including substances used as nutrients by microorganisms and those which may be injurious to their activities; (2) ability to absorb water in large amounts; (3) flocculation and deflocculation phenomena of colloids, including bacterial cells considered as colloids; (4) the "sol" and "gel" states of the colloids; (5) modification of soil conditions, such as reaction.

Colloids are coagulated, or flocculated, by means of electrolytes; the univalent ions have a less intensive effect than the bivalent and the latter less than the trivalent. Clay is an electro-negative colloid and can be flocculated by positively charged ions; at a certain point it is deflocculated by the negatively charged hydroxyl ions. This deflocculation may be due to the lessened solubility of the di- and tri-valent cations in the soil rather than to the direct effect of the hydrogen-ion concentration.<sup>20</sup> Of particular importance is the action of calcium in flocculating colloids. Sodium salts, notably sodium carbonate, serve to deflocculate the soil colloids, although a sufficient concentration of sodium ions may even cause flocculation. The flocculation of soil particles is similar to the flocculation of suspensoid sols and is amenable

<sup>19</sup> Loeb, J. *Proteins and the theory of colloidal behavior*. McGraw-Hill. New York. 1922; see Mattson, S. *Soil Sci.*, **28**: 179-220, 373-409. 1929.

<sup>20</sup> Dayhuff, W. C. and Hoagland, D. R. *Soil Sci.*, **18**: 401-408. 1924.

to the isoelectric theory, except in the case of lime.<sup>21</sup> Soil organic matter has a protective effect on the flocculation of clay.<sup>22</sup>

Calcium carrying a double positive charge precipitates the negative soil colloids, bringing about a change in the plastic properties of the soil. The soil structure is thus changed entirely; the resistance to penetration of moisture is reduced, an increase in pore space is brought about, and there is an increase in the water holding capacity of the soil. This improves the physical condition of the soil as a medium for the activities of microorganisms. Drying of the soil also causes a coagulation of the colloids, the change produced thereby being reversible.

The soil organic compounds contain reversible and irreversible colloids. The addition of lime brings about an increase in the water-soluble carbon compounds of the soil which favorably influences bacterial activities (in addition to favorable effect of reaction). This leads to a greater decomposition of the soil organic matter with the formation of  $\text{CO}_2$ ,  $\text{NH}_3$ , nitrates and soluble phosphates. Thus the application of lime leads to a neutralization of the soil acids, an increase in the decomposition of the soil organic matter, greater liberation of  $\text{CO}_2$ , mineralization of the organic matter, absorption of bases and increase in colloidal matter.<sup>23</sup>

Van Bemmelen<sup>24</sup> was the first to show that soil humus plays an important part in the absorption of both basic and acid radicals from the soil solution; this process of absorption was found to be similar in nature to that of an artificial calcium-aluminum silicate. Different forms of absorption in the soil are often recognized:<sup>25</sup> (1) biological absorption or the assimilation of the anions or cations by microorganisms; (2) mechanical absorption, or the mere mechanical retention of particles suspended in water; (3) physical, or surface adsorption, which may be positive or negative, depending on the fact as to whether the substance decreases or increases the surface tension of the dispersion medium; (4) physico-chemical, or adsorption in the narrow sense,

<sup>21</sup> Comber, N. M. *Trans. Faraday Soc.*, **17**: 349. 1922; *Jour. Agr. Sci.*, **10**: 425-436. 1930; Mattson, S. E. *Inaug. Diss.* Breslau. 1922; *Kolloid Chem. Beihefte*, **14**: 227-313. 1922.

<sup>22</sup> Wolkoff, M. I. *Soil Sci.*, **1**: 585-601. 1916; Oden, S. *Jour. Landw.*, **67**: 177-208. 1919.

<sup>23</sup> Thær, W. *Göttingen*. 1910 (*Centrbl. Bakt.* II, **32**: 271-274. 1912).

<sup>24</sup> van Bemmelen, J. M. *Landw. Vers. Sta.*, **35**: 69-136. 1888; *Die Absorption*. Dresden. Steinkopff. 1910.

<sup>25</sup> Gedroiz, K. K. *Zhur. Opit. Agron.*, **19**: 269-322. 1918; *Leningrad*. 1922.

which consists in the exchange of bases between the added salt and the zeolitic or aluminosilicate (and humic) complex of the soil; and finally (5) chemical absorption, or the chemical interaction between two substances giving difficultly soluble compounds, as in the formation of calcium phosphate from the carbonate and soluble phosphate. Certain investigators, however, do not take the view that any sharp differentiation exists between chemical and physical reactions, which may all be due to electrical forces differing only in degree; this applies especially to the exchange of bases in the soil.

In proportion to their total mass, colloids exhibit a remarkable power of adsorption because of the large surface that they possess. Adsorption increases with the concentration of the solute. The absorption of the ammonium ion by soils or by hydrous silicates from a solution of an ammonium salt follows the laws of adsorption.<sup>26</sup> The same was found to hold true for the adsorption of other bases. Among the common anions the  $\text{PO}_4$ -ion is adsorbed under all conditions of reactions, while the  $\text{Cl}$ ,  $\text{SO}_4$  and  $\text{NO}_3$  ions are adsorbed from acid solutions only, especially by soils having a high sesquioxide content.<sup>27</sup> When bases are absorbed by soil, they displace an equivalent amount of another base which is combined in the soil either with the inorganic zeolites or with the organic compounds.

The adsorption of dyes by soils, which depends upon the surface of the soil, has been used for the estimation of colloids of the soil by assuming that only the colloids take part in this process.<sup>28</sup>

Colloids play an important part in making the soil a favorable medium for the growth of microorganisms, by absorbing the soluble fertilizing elements added to or produced in the soil and by their buffering properties in preventing rapid changes of soil reaction. The growth of bacteria was found<sup>29</sup> to be a function of the soil surface; in culture media colloidal silicic acid and its compounds, as well as colloidal ferric and aluminum hydrates and humus stimulate nitrogen fixation by *Azotobacter*, possibly by absorbing nitrogen gas. A colloid (like soil

<sup>26</sup> Wiegner, G. Jour. Landw., 60: 111-150, 197-222. 1912.

<sup>27</sup> Mattson, S. Proc. First Intern. Congr. Soil Sci., (1927) 2: 199-211. 1928.

<sup>28</sup> A detailed discussion of the subject of absorption is found in the following papers: Whitney, M. and Cameron, F. U. S. Dept. Agr., Bur. Soils, Bul. 22. 1903; Bul. 23. 1904; Cameron, 1911 (p. 557), p. 61; Wiegner, G. Jour. Landw., 61: 11-56. 1913; Prescott, J. Jour. Agr. Sci., 8: 110-130. 1916; Gedroiz, 1922 (p. 553); Fischer, E. A. Trans. Faraday Soc., 17: 305-316. 1922; Mattson, 1922 (p. 553).

<sup>29</sup> Söhnngen, N. L. Centrbl. Bakt. II, 38: 621-647. 1913.

extract, gelatin, etc.) quickly shortens the period necessary for germination of the spores of *Bac. amylobacter* in a nutrient solution.<sup>30</sup> In the presence of 0.25 to 1.0 per cent gelatin, the period of incubation, from inoculation to beginning of fermentation, was reduced from fifty-one days in a nutrient solution free from colloids to three days. In the presence of a colloid, a clear zone is found to surround the spores; this zone is absent in a suspension of spores in a colloid-free solution. The shortening of the period necessary for spore germination depends on the dispersion of the colloid and is explained chiefly by adsorption phenomena.

Bacteria are adsorbed by various colloids as well as by sand.<sup>31</sup> Since soils contain an abundance of substances in a colloidal condition, it is but natural to expect a marked influence upon the bacteria. The following method can be used for the study of this phenomenon.<sup>32</sup> One cubic centimeter of a bacterial culture is added to 9 cc. of water and the mixture placed in a flask containing 5 grams of soil. After shaking for one minute, the soil is allowed to settle for ten minutes. The number of bacteria is then determined in the suspension both by plating and by direct microscopic examination. It was found that pure sand has little adsorptive action. Some bacteria, like *Bac. mycoides*, *Bact. prodigiosum* and *Staph. pyogenes*, are adsorbed rapidly and completely (80 to 98 per cent); other bacteria, like *Bact. coli*, are only weakly adsorbed (10 to 20 per cent). Adsorption of the bacteria was found to lead to a diminution not only in numbers but also in their chemical activities. Decomposition of organic matter in the soil seems to be carried out largely by the unadsorbed bacteria, probably due to the lower oxygen tension upon the soil colloidal particles. Adsorption does not diminish the action of anaerobic bacteria upon organic matter in the soil.

The absorption of inorganic materials by microorganisms is quite marked, some bacteria and fungi possessing a greater absorptive power than higher plants per unit of cell substance.<sup>33</sup>

<sup>30</sup> Lantzsch, 1921 (p. 544); Dorner, 1924 (p. 172). Further information on the influence of colloids upon the activities of microorganisms is given by Plotho, O. *Biochem. Ztschr.*, **110**: 33. 1920; Schade, H. *Die Naturw.*, **9**: 89-92. 1921.

<sup>31</sup> Eisenberg, P. *Centrbl. Bakt. I, Orig.*, **81**: 72-104. 1918; Frey, W. and Erismann, H. *Centrbl. Bakt. I*, **88**: 306-336. 1922.

<sup>32</sup> Dianowa, E. W. and Woroshilowa, A. A. *Nautchno-Agron. Zhur. No. 10*. 1925; Chudiakow, N. N. *Centrbl. Bakt. II*, **68**: 345-358. 1926.

<sup>33</sup> Beijerinck, M. W. *Centrbl. Bakt. II*, **29**: 161-166. 1911; Stoklasa, J. *Chem. Ztg.*, **35**: 1425. 1911; Labes, R. *Biochem., Ztschr.*, **130**: 1-13. 1922; Beard, E. and Cramer, W. *Proc. Roy. Soc. B*, **88**: 575. 1915; **98**: 584. 1915.

*Soil solution.* The water present in the soil and added through rainfall dissolves some of the soil constituents. If the soil conditions were stable, the solution would soon become saturated. Constant evaporation, rainfall, change in weather conditions, development of acids by microorganisms, absorption of inorganic elements by higher plants, and many other changing conditions, cause an unceasing fluctuation in the composition and concentration of the soil solution. The osmotic pressure of the soil solution varies<sup>34</sup> from 0.1 to 1 atmosphere in most soils to 4.5 to 16.5 atmospheres in soils with low moisture content. In normal soils, the concentration of the soil solution ranges between 0.1 and 1 atmosphere, depending on the rainfall, system of fertilization and plant growth.<sup>35</sup>

TABLE 53  
*Composition of soil solution*

NATURE OF SOIL	MOISTURE IN SOIL	PARTS PER MILLION OF SOIL SOLUTION			
		K	PO <sub>4</sub>	Ca	N
Fine sand.....	29.74	24.1	5.2	30.6	3.1
Loam.....	37.80	71.1	12.2	68.2	3.2
Clay.....	24.50	44.8	4.6	42.9	6.1
Peat.....	132.90	50.1	2.5	183.8	17.1

The soil solution contains calcium nitrate and bicarbonates, some organic matter, Na, Mg, Si, Cl, SO<sub>4</sub>, small amounts of K, and traces of ammonia and phosphates, as shown in table 58.<sup>36</sup>

It is from this solution that the microorganisms obtain a large part of their food and in it they leave their waste products. The colloidal nature of the soil has an important bearing upon the nature of the soil solution. The rates of solubility, decomposition of organic matter, carbon dioxide production, nitrification, absorption of the soluble constituents by plants, microorganisms and soil particles, all have an important bearing upon the nature and concentration of the soil solution. At a given moisture content, the rate of formation of soluble material increases with the temperature.<sup>37</sup> At higher temperatures,

<sup>34</sup> Bouyoucos, G. J. and McCool, M. M. Mich. Agr. Exp. Sta. Tech. Bul. 24. 1915; 27. 1916; 31. 1910.

<sup>35</sup> Hoagland, D. R. Jour. Agr. Res., 12: 369-395. 1918.

<sup>36</sup> Morgan, J. F. Soil Sci., 3: 531-545. 1917.

<sup>37</sup> McCool, M. M. and Whiting, L. C. Soil Sci., 11: 233-247. 1921.

optimum moisture conditions tend to bring greater amounts of material into solution than are found under saturated water conditions; with lower temperatures, the opposite effect was observed. Below two feet, peat soils are very inactive, the ability of producing soluble materials decreasing regularly from the surface to the water level, indicating that aeration greatly influences this process.

A definite correlation was found<sup>38</sup> between bacterial activities in the soil and the thickness of the moisture film. The optimum thickness of the film in the case of *Bac. mycoides* was found to be between 20 and 40 microns. This film was obtained in sand of 1 mm. diameter at a moisture content of about 10 per cent. In arable soils with a grain size not more than 0.1 mm., it would require more than 50 per cent of moisture to produce the optimum film thickness.

Lowering of the freezing point and conductivity of the soil<sup>39</sup> can also be used as indices of changes in the composition of the soil solution. Pantanelli<sup>40</sup> suggested the use of electrolytic conductivity of soils for studying the course of solubilization of soil constituents by microorganisms; this was found, in most cases, to vary with the bacterial content of the soil. It is doubtful whether the actual concentration of the soil solution can be determined by the electrical bridge, since in most of these measurements an excess of water is added.

*Soil reaction and microbiological activities.* The nature and quantity of substances present in the soil in a colloidal condition, which act as buffers or are capable of combining with acids and bases, the nature and amount of bases present in the soil, either in an adsorbed condition or in the form of carbonates, influence the reaction of the soil, the medium in which the microorganisms live and act. Soil acidity may be due either to free organic and inorganic acids, which liberate hydrogen ions, or to a non-saturation of the soil organic and inorganic complexes with bases, which results in a replacement of the base by hydrogen.<sup>41</sup>

When neutral salts are added to a soil, the cations are adsorbed, replacing the hydrogen ions, thus making the soil even more acid.<sup>42</sup>

<sup>38</sup> Rahn, 1913 (p. 547).

<sup>39</sup> Davis, R. O. E. and Bryan, H. U. S. Dept. Agr., Bur. Soils, Bul. 61. 1910; König, J., Hasenbäumer, J. and Glenk, K. Landw. Vers. Sta., 80: 491-534. 1913.

<sup>40</sup> Pantanelli, E. Centrbl. Bakt. II, 42: 439-443. 1915.

<sup>41</sup> Gedroiz, K. K. Zhur. Opit. Agron., 22: 3-27. 1924; Hissink, D. J., and Van der Spek, J. Verslag. Land. Onderzoek, Rijksland., 27: 146-161. 1922.

<sup>42</sup> Cameron, F. K. The soil solution. Easton, Pa. 1911; Kappen, H. Landw. Vers. Sta., 89: 39-80. 1916; 96: 277-307. 1920; Wrangell, M. Landw. Vers. Sta., 96: 209-255. 1920; Harris, J. E. Mich. Agr. Exp. Sta., Tech. Bul. 19. 1914; Jour. Phys. Chem., 18: 355. 1914.



It has also been suggested<sup>43</sup> that acidity in well aerated soils is due to the hydrolysis of silicates; the bases are removed by plants or soil water, while the acid silicates are left behind.

Definite concentrations of free hydrogen-ions are found in the soil, and can be measured electrometrically and colorimetrically.<sup>44</sup> A pH of 3.7 is the extreme value usually obtained for mineral acid soils, while certain highmoor peats may be even more acid (pH 3.2). The highest pH values are reported for alkali soils, namely pH 9.7 to 10.0. Fertile soils, however, usually give a range of pH values of 6.0 to 7.5.

Soils are usually well buffered over considerable ranges of hydrogen-ion concentrations.<sup>45</sup> By adding acid or base to a soil and titrating the resulting hydrogen-ion concentrations, a linear titration curve is obtained which can serve as an index of the buffer content of the soil.

TABLE 59  
*Influence of different nitrogenous fertilizers upon the reaction of the soil*  
100 mgm. nitrogen added to 100 grams of soil

INCUBATION	UREA 220 MGm.	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 500 MGm.	NaNO <sub>3</sub> 660 MGm.
	pH	pH	pH
Start.....	6.45	6.45	6.45
2 days.....	7.60	6.90	6.85
35 days.....	6.25	6.20	6.80
50 days.....	5.70	5.40	6.60
76 days.....	5.35	5.10	6.55

The slopes of the curve vary with different soils according to their buffer content. It has been suggested<sup>46</sup> that, although there may be no correlation between the reaction of an acid soil and crop growth, there is a definite correlation between the latter and the buffer content of the soil.

<sup>43</sup> Truog, E. Jour. phys. Chem., 20: 457-484. 1916.

<sup>44</sup> Sharp, L. T. and Hoagland, D. R. Jour. Agr. Res., 7: 123-145. 1916; Soil Sci., 7: 196-200. 1919; Gillespie, L. Jour. Wash. Acad. Sci., 6: 7-16. 1916; Soil Sci., 4: 313-319. 1917; Michaelis, L. Die Wasserstoffionenkonzentration. Berlin. 1922; Clark, 1928 (p. XVIII); Christensen, H. and Jensen, S. T. Intern. Mitt. Bodenk., 14: 1-26. 1924; Bayer, L. D. Soil Sci., 21: 167-180. 1926; Bijlman, E. Bull. soc. Chim. (4), 61-62: 213-286. 1927.

<sup>45</sup> Charlton, J. Mem. Dept. Agr. India, 7: 101-121. 1924; Jensen, S. T. Ber. Statens Forsogs. i. Plantenk, 1924; Arrhenius, O. Jour. Amer. Chem. Soc., 44: 521-524. 1922; Trans. Second Comm. Intern. Soc. Soil Sci. Groningen. 1926; Maiwald, K. Kolloid chem. Beih., 27: 251-346. 1928.

<sup>46</sup> Arrhenius, O. Soil Sci., 14: 21-26, 223-232. 1922.

The concentration of carbon dioxide is of great importance in such a system of measurement, especially when a relatively poorly buffered soil extract is used. The actual soil solution surrounding the absorbing membrane of the plant roots may be slightly acid, although the soil suspension gives an electrometric measurement of pH 7.0 and above. Titratable acidity has much wider ranges of variation due to differences in buffer content. A peat or clay soil may have the same hydrogen-ion concentration as a sandy soil, but the titration (or lime requirement) of the first two soils will be much higher due to the greater buffer content.

When nitrogen-poor organic matter is added to the soil in the form of green manure or plant stubble, the first stage of decomposition may result in the formation of various organic acids, particularly in the absence of free calcium carbonate. If aeration, temperature and reaction favor further decomposition of the organic acids thus formed, a change in reaction to alkalinity takes place, due to the formation of  $\text{CO}_2$  and carbonates.<sup>47</sup> The reaction of the soil as a result of application of fertilizer may change, according to the nature of the biological transformation of the fertilizer. Urea, for example, causes the soil reaction first to become alkaline because of the formation of ammonia and then acid because of the oxidation of the ammonia to nitric acid, as shown in table 59.<sup>48</sup> The soil reaction is also influenced by the moisture content of the soil, application of fertilizers, green manures, stable manures,<sup>49</sup> plants grown and other factors.<sup>50</sup>

The reaction of the soil has a definite influence upon the activities of various microorganisms and upon the distribution of the microflora and microfauna in the soil. An acid soil favors the development of fungi and is distinctly injurious to the growth of certain groups of bacteria, like *Azotobacter*, which has a limiting reaction at pH 6.0. Nitrifying bacteria are limited in their activities to a maximum acid range

<sup>47</sup> Coville, F. V. *Smithson. Report for 1913*: 333-343. 1914; Ayers, S. H. and Rupp, P. *Jour. Inf. Dis.*, **23**: 188-216. 1924.

<sup>48</sup> Brioux, Ch. *Compt. Rend. Acad. Sci.*, **179**: 914-917. 1924.

<sup>49</sup> Agnides, E. *Intern. Rev. Sci. Pract. Agr.* (2) **4**: 294-306. 1926.

<sup>50</sup> Plummer, J. K. *Jour. Agr. Res.*, **12**: 19. 1918; Knight, H. G. *Jour. Ind. Engin. Chem.*, **12**: 559. 1920; Hardy, F. *West Indian Bul.* **19**: 37-85. 1921; Salter, R. M. and Morgan, M. F. *Jour. Phys. Chem.*, **27**: 117-140. 1923; Fischer, E. A. *Jour. Agr. Sci.*, **11**: 19-44. 1921; *Sci. Progr.*, **16**: 408. 1922; Conner, S. D. *Jour. Agr. Res.*, **15**: 321. 1918; Morse, F. W. *Jour. Ind. Engin. Chem.*, **10**: 125. 1918; Atkins, W. R. G. *Sci. Proc. Roy. Dublin Soc.*, **16**: 369-413, 414-426, 429-434. 1922; Kappen, H. and Zapfe, M. *Landw. Vers. Sta.*, **90**: 321-374. 1917.

TABLE 60

*Optimum and limiting reactions for the activities of microorganisms*

ORGANISMS	ACID MAXIMUM	OPTIMUM	ALKALI MAXIMUM	AUTHOR
	pH	pH	pH	
<i>Nitrosomonas</i> .....	3.9	7.7-7.9	9.7	Gaarder and Hagem
<i>Nitrobacter</i> .....	3.9	6.8-7.3	13.0	Meek and Lipman
Nitrification in soil.....	3.5	6.5-7.5	>11.9	Gerretsen, Waksman
<i>Thiobacillus denitrificans</i> ....	5.0	7.0-9.0	10.75	Trautwein
<i>Th. thiooxidans</i> .....	1.0>	2.0-4.0	6.0(?)	Waksman and Starkey
<i>Cytophaga hutchinsoni</i> .....	5.7-6.0			Dubos, Jensen
<i>Bac. pycnoticus</i> .....	5.2	6.8-8.7	9.2	Ruhland
<i>Bac. amylobacter</i> .....	5.7>	6.9-7.3		Dorner
<i>Azotobacter</i> .....	5.6-6.0	6.5-7.8	8.8-9.2	Gainey, Johnson and Lipman, Yamagato and Itano, Stapp
<i>Bact. radicola</i> of				
Medicago and Melilotus...	5.0			
Pisum and Vicia.....	4.8			
Trifolium and Phaseolus..	4.3	6.5-7.2	11.0	Fred and Davenport, Fred and Loomis, Bryan <sup>a</sup>
Soja.....	3.4			
Lupinus.....	3.2			
<i>Bact. coli</i> .....	4.4	6.5	7.8	Dernby
<i>Bact. vulgare</i> .....	4.4	6.5	8.4	Dernby
<i>Bact. pyocyaneum</i> .....	5.6	6.8	8.0	Dernby
<i>Bact. stutzeri</i> .....	6.1	7.0-8.2	9.6-9.8	Zacharowa
<i>Bac. subtilis</i> .....	4.2	7.5-8.5	9.4	Itano
<i>Bac. putrificus</i> .....	5.8	6.8	8.5	Dernby
<i>Act. scabies</i> .....	4.8-5.0	6.5-7.5	8.7	Gillespie, Waksman
<i>Mucor glomerula</i> .....	3.2-3.4		8.7- 9.2	
<i>Asp. terricola</i> .....	1.6-1.8		9.0- 9.3	
<i>Pen. italicum</i> .....	1.6-1.8		9.1- 9.3	Johnson
<i>Fus. oxysporum</i> .....	1.8-2.0		9.2-11.1	
<i>Asp. niger</i> .....	1.2	1.7-7.7		Terroine and Wurmser
<i>Gibberella saubinetii</i> .....	3.0	4.8-9.4	11.7	MacInnes
Spore germination of fungi..	1.5-2.5	3.0-4.0		Webb
Protozoa:				
<i>Paramoecium</i> }.....	3.3-3.9	7.0-7.4	9.0	Cutler and Crump
<i>Colpidium</i> }				

<sup>a</sup> See also Hosoda, K. Iothosi Nog. Kwaiho., 1: 89-105. 1928.

of pH 4.0 to 4.6; *Bact. radiculicola* has its limiting acid reaction at pH 3.4 and pH 6.0, depending on the strain. Cellulose decomposing bacteria are inhibited by a reaction of pH 5.7-6.0, although, here as well, different species will behave differently. Actinomyces are inhibited in growth by reactions more acid than pH 4.8; this fact is utilized for the control of *Act. scabies* causing potato scab in the soil. The application of lime to an acid soil has a favorable influence upon the bacteriological activities; the growth of microorganisms may even be stimulated more than that of higher plants grown upon the soil.<sup>52</sup>

Acids affect the activities of microorganisms not merely by creating a favorable or unfavorable hydrogen-ion concentration, but also through the undissociated part of the molecule.<sup>53</sup>

The optimum and limiting reactions of some typical soil organisms are indicated in table 60. According to Olsen,<sup>54</sup> ammonia formation will proceed in soils whose pH values lie between 3.7 and 9.0, but the process is most active at pH 7.0-8.5. Nitrification can proceed in soils of pH 3.7-8.8. At pH 4.0-8.0, nitrate formation is limited only by the rapidity of ammonia formation in soil.

*The soil atmosphere.* The soil atmosphere is a mixture of gases which change constantly in composition, chiefly because of biological activities and also to some extent because of chemical processes. The composition of this atmosphere depends upon the amount and nature of the organic matter and upon the environmental conditions. During dry seasons, when oxidation of the organic matter is low, the soil gases are rich in oxygen and poor in CO<sub>2</sub>. After heavy rains, the oxygen content rapidly diminishes and the CO<sub>2</sub> content increases because of the active oxidation of the soil organic matter. The nitrogen content of the atmosphere of aerated soils does not vary appreciably and is not affected either by the assimilation of nitrogen by the bacteria or by its liberation from the decomposition of nitrogenous compounds of the soil.

The amount of carbon dioxide in fallow land is smaller than in soil which is vegetated. The atmosphere of soil freshly treated with farm manure or green manure contains a high proportion of CO<sub>2</sub> and a relatively low concentration of oxygen. The actual CO<sub>2</sub> content of the soil atmosphere thus depends upon a number of factors, including (1)

<sup>52</sup> Brown, P. E. Centrbl. Bakt. II, 34: 148-172. 1912; 35: 234-248. 1912; Waksman, 1922 (p. 695).

<sup>53</sup> Hall, I. W. and Fraser, A. D. Jour. Pathol. Bact., 25: 19-25. 1922.

<sup>54</sup> Olsen, C. Compt. Rend. Lab. Carlsberg, 17: 1-21. 1928.

CO<sub>2</sub> production from the decomposition of organic matter and by the chemical interaction between carbonates and acids; (2) diffusion of the CO<sub>2</sub> in the soil atmosphere; (3) assimilation of CO<sub>2</sub> by plants.<sup>55</sup> A large part of the CO<sub>2</sub> is present in the soil solution. Diffusion of CO<sub>2</sub> into the atmosphere and of oxygen into the soil is very rapid at a depth of six inches,<sup>56</sup> as shown in table 61.

The soil atmosphere shows much greater fluctuations in composition than atmospheric air. On the average, the air of arable soil was found<sup>57</sup> to contain 0.15 to 0.65 per cent CO<sub>2</sub> and 20.6 per cent oxygen. From November to May the curves for CO<sub>2</sub> follow closely those of the soil temperature; from May to November they follow rainfall and to a less extent the soil temperature curves. The favorable effect of rainfall is

TABLE 61  
*Composition of gas in variously treated soils*

	AVERAGES OF SEVERAL DETERMINATIONS, IN PER CENT				
	Fallow land		Gases near roots of corn	Green manured land	Swamp rice land
	Before rainfall	After rainfall			
Nitrogen.....	78.05	78.83	80.15	79.18	85.59
Oxygen.....	20.40	19.26	9.00	7.71	0.54
Carbon dioxide.....	0.58	0.95	9.11	12.03	4.42
Hydrogen.....	None	None	0.73	0.07	6.42
Methane.....	None	None	None	None	2.81
Argon.....	0.977	0.955	1.010	1.003	0.893
N.....					
A.....	80.0	82.5	79.5	78.8	95.7

believed to be due to the dissolved oxygen brought down. With an increase in organic matter content of soil and in the presence of a growing crop, the CO<sub>2</sub> content increases (p. 607). The gases formed under anaerobic conditions, as in swamp rice soils, consist largely of methane, hydrogen, carbon dioxide and nitrogen.<sup>58</sup>

<sup>55</sup> Attention need only be called here to the early contribution of Boussingault and Lewy. Ann. Chim. Phys. (3 ser.). 37. 1853. For more recent investigations, see Romell, L. G. Meddel. fran Statens Skogsförsöks. H. 19. 1922; Lundegårdh, H. Der Kreislauf der Kohlensäure in der Natur. G. Fischer, Jena. 1924.

<sup>56</sup> Leather, J. W. Mem. Dept. Agr. India, Chem. Ser., 4: 85-134. 1915.

<sup>57</sup> Russell, E. J. and Appleyard, A. Jour. Agr. Sci., 7: 1-48. 1915; 8: 385-417. 1917; see also Potter, R. S. and Snyder, R. S. Iowa Agr. Exp. Sta. Res. Bul. 39. 1916.

<sup>58</sup> Harrison, W. H. and Aiyer, P. A. S. Mem. Dept. Agr. India, Chem. Ser., 3: 65-106. 1913.

An increase in atmospheric pressure brings about first an increase in the activities of certain microorganisms, such as the autotrophic bacteria, and then a slackening.<sup>59</sup>

The CO<sub>2</sub> of the soil atmosphere was found<sup>60</sup> to be a more important source of carbon for the growth of plants than the CO<sub>2</sub> of the air. Plants thus depend entirely upon the activities of the microorganisms in the soil for their CO<sub>2</sub>, which is liberated largely as a result of the decomposition of the soil organic matter.

*Soil temperature.* The temperature of the soil is affected by climate, season of year, chemical and mechanical composition of soil, topography, and treatment. In the spring of the year, fine-grained soils containing a large amount of water warm up more slowly than coarse-grained soils containing a relatively small amount of moisture. The heat conductance of the specific soil constituents is of importance; cultivation of the soil plays also an important rôle, by influencing the rate of evaporation. In general, sandy soils and sandy loams warm up more quickly in spring than heavy clay and clay loam soils; microbial activities are, therefore, sooner accelerated in the spring in the first types of soil than in the second.<sup>61</sup>

The colloidal condition of the soil is modified in the temperate climates by the action of frost during the winter, so that, when the soil finally warms up in the spring and loses the excess moisture, a rise in biological activities takes place.<sup>62</sup> In the summer months there is a drop to normal which is undoubtedly due to the fact that the available energy has been largely used up and the soil may lack in sufficient moisture. In the autumn there is another rise in biological activities which is probably due to the addition of plant residues. The drop in winter is due to the low temperature. Bacterial activities are not, however, entirely suspended during the colder seasons of the year. The activities of some of the most important soil organisms become marked at temperatures above 10°C. with an optimum at 25°. A detailed study of the influence of temperature upon the biological activities in the soil is given elsewhere (p. 747).

<sup>59</sup> Berghaus, W. H. Arch. Hyg., 62: 172-200. 1907; Chlopin, G. W. and Tammann, G. Ztschr. Hyg., 45: 171-204. 1903.

<sup>60</sup> Lundegårdh, H. Klima und Boden. Fischer, Jena. 1930; Reinau, E. Praktische Kohlensäuredüngung in Gärtnerei und Landwirtschaft. J. Springer. Berlin. 1927.

<sup>61</sup> Lipman, J. G. Microbiology of soil. In Marshall's Microbiology. 3d Ed. 345-427. 1921.

<sup>62</sup> Müntz, A. and Gaudechon, H. Ann. Sci. Agron. (4), 2: 1-15. 1913.

*Growth of microorganisms in soil in pure and mixed culture.* There is no method available for sterilizing the soil without changing its physical and chemical properties. The common method employed at present consists in heating the soil in flowing steam for thirty minutes on seven consecutive days,<sup>63</sup> or at high pressure (15 pounds) for two hours. Both of these treatments cause a decided change in the physical and chemical soil conditions which results in an increase in the available organic matter. Sterile soil forms an excellent medium for the development of various bacteria and other microorganisms.<sup>64</sup> A number of organisms, such as *Azotobacter* or *Cl. pastorianum*, regain their vigor of fixing nitrogen, when cultivated in sterile soil.<sup>65</sup>

However, from processes carried out by pure cultures of microorganisms grown in sterile soil we cannot determine what actually occurs in normal soils. Not only is the nature and composition of the culture medium completely changed by sterilization of soil, but the various antagonistic and associative influences which are active in normal soils (p. 369) are eliminated. This can be readily illustrated by the following instances. An organism belonging to the *Bac. mesentericus* group was found<sup>66</sup> capable of dissolving and clarifying cultures of *Bact. coli*, *Staphylococcus*, and other bacteria, a phenomenon which may not take place in pure culture. The inhibitive effects of filamentous fungi, especially of ascomycetes, on the growth of microorganisms has been commonly observed;<sup>67</sup> this may be due to exhaustion of nutrients or to the formation of some toxic products during growth.<sup>68</sup> Under natural conditions, the members of the soil population have adjusted themselves to the antagonistic and stimulating influences exerted by one upon the other. This population is made up of certain common organisms, which are universally distributed and which are not very sensitive to changes in environmental conditions. Among the bacteria, various heterotrophic spore-formers (*Bac. cereus*, *Bac. mycoides*, *Bac. mesentericus*, etc.) and non-spore formers (*Bact. fluorescens*, *Bact. caudatum*, *Bact. radiobacter*, etc.), the nitrogen-fixing *Azotobacter* and *Cl. pastorianum*, the nitrifying organisms, etc., are of universal occurrence, limited only by certain specific soil conditions, such as reaction.

<sup>63</sup> Eckelmann, I. Centrbl. Bakt. II, 48: 140-178. 1918.

<sup>64</sup> Barthel, C. Centrbl. Bakt. II, 48: 340-349. 1918.

<sup>65</sup> Bredemann, 1909 (p. 104).

<sup>66</sup> Kimmelstiel, P. Centrbl. Bakt. I, Orig. 89: 113-115. 1922.

<sup>67</sup> Porter, C. L. Amer. Jour. Bot., 11: 168-188. 1924.

<sup>68</sup> Liesegang, R. Centrbl. Bakt. II, 51: 85-86. 1920.

They were reported from all soils, from East and West, North<sup>69</sup> and South. The most common soil protozoa, including the amoebae, flagellates and ciliates, are universally distributed in the soil.<sup>70</sup> The common soil fungi, including species of *Zygorhynchus*, *Trichoderma*, *Aspergillus*, *Penicillium*, etc., have been isolated from various soils coming from different parts of the world. The same is true of algae and other soil microorganisms.

We are thus fully justified in speaking of a soil population and may even accept the idea of an Edaphon, as suggested by Francé, although his conclusion that the edaphon is an indicator of soil fertility may not be fully justified.<sup>71</sup> Under very specific soil conditions, however, a specific soil flora and fauna may develop, differing quantitatively and qualitatively from that found in ordinary field and garden soils. It is sufficient to mention forest soils with their specific fungus flora rich in Basidiomycetes, peat bogs with a specific flora of anaerobic bacteria, alkali soils with a highly limited micro-population, in which actinomyces and aerobic bacteria predominate. It has been suggested<sup>72</sup> that alkali soils profit by the improvement of their physico-chemical condition not only in respect to the growth of higher plants but also from a change in their microflora. The changes produced in the micropopulation of peat soils as a result of drainage, liming and cultivation is discussed elsewhere (p. 663).

<sup>69</sup> Barthel, Chr. Saertr. Meddl. Gronland, 64. 1922.

<sup>70</sup> Sandon, 1924-1927 (p. 313).

<sup>71</sup> Francé, R. H. Das Edaphon, Stuttgart. 1921; Fischer, H. Int. Mitt. Bodenk., 13: 192-200. 1923.

<sup>72</sup> Bokor, R. Erd. Kiserletek., 30: 1-25. 1928.



## CHAPTER XXIII

### TRANSFORMATION OF MINERALS IN THE SOIL

*Nature of mineral transformation by microorganisms.* Among the mineral elements of plant food which are subject directly or indirectly to the action of microorganisms, the following may be included: phosphorus, sulfur, potassium, calcium, magnesium, iron, sodium, manganese, also chlorine, aluminum, zinc and silicon. The transformation of the elements and compounds of carbon, nitrogen, hydrogen and oxygen, both in organic and inorganic combinations, are considered in detail elsewhere. The above minerals are transformed in the soil by different processes:

1. Mineral elements (S, Fe, Mn, etc.) or their inorganic compounds may be used by certain bacteria as sources of energy.

2. Certain salts (nitrates, sulfates, etc.), rich in oxygen, may be used, under conditions favoring anaerobiosis, as sources of oxygen.

3. The transformation of minerals present in the soil in the form of complex organic compounds. When the bodies of plants, animals and microorganisms are decomposed or mineralized by soil microorganisms, a part of the minerals is liberated in the form of inorganic compounds and a part may be reassimilated.

4. The assimilation of minerals by microorganisms. In the presence of available energy, simple inorganic salts, especially phosphates, potassium salts and sulfates, are converted into complex organic compounds. This leads to a temporary removal of the soluble salts; most of these materials are again made available upon the death and decomposition of the microbial cells, as described in the third process.

5. The indirect transformation of minerals in the soil by products of metabolism of microorganisms. The action of carbon dioxide, organic and inorganic acids upon carbonates, phosphates and silicates is largely due to the change in the  $H^+$  or the  $OH^-$  concentrations of the soil. This is true also of the interaction between insoluble phosphates and the so-called "humic acids."<sup>1</sup>

Phosphates, sulfates and salts of potassium, calcium and magnesium (iron salts in smaller amounts), are the most important compounds in the metabolism of all microorganisms. Large quantities of these are taken from the soil solution and synthesized into microbial protoplasm. In the decomposition of plant residues and animal manures, phosphates

<sup>1</sup> Baumann, A. and Gully, E. Mitt. K. Bayr. Moork. 4: 31-156, 1910; Niklas, H. Diss. München. 1912.

and potassium salts are made readily soluble; calcium and magnesium, however, are present in a less soluble form.<sup>2</sup> The decomposition and synthesis of organic matter in the soil takes place constantly and leads to constant changes in the amount of available minerals in the soil. It has been suggested<sup>3</sup> that fungi take a more active part in the decomposition of mineral soil constituents than root secretions of higher plants. Various bacteria also play important rôles in the process. The fungi and algae, as well as other groups of microorganisms, store away considerable quantities of soluble minerals in the form of microbial protoplasm due to the extensive growth of the organisms in the presence of available energy.

*Decomposition of rocks and rock constituents by microorganisms.* Not only the mineral constituents of normal soils but rocks as well may undergo disintegration and degradation through the action of microorganisms. Autotrophic bacteria obtain their carbon from the carbon dioxide of the atmosphere, and their energy from inorganic substances of the soil or from ammonia formed by electrical discharges and rainfall; algae utilize the photosynthetic energy of the sun. The various inorganic and organic acids formed by these organisms exert solvent action upon the rocks.

The chemosynthetic assimilation of CO<sub>2</sub>, fixation of nitrogen, and denitrification are considered<sup>4</sup> as the three most primitive activities of microorganisms in the development of life upon this planet. It has been demonstrated<sup>5</sup> that various algae, particularly the Cyanophyceae, exert a corroding effect upon stones. Diatoms transform aluminum silicates into hydrated aluminum oxide.<sup>6</sup> Müntz and others<sup>7</sup> suggested in 1890 that bacteria are concerned in rock decomposition, their action being confined not only to the surface but often entering into the depth of the rock mass; nitrifying organisms were always demonstrated in weathering rocks. At the growing margins of crustaceous lichens, a definite disintegration of the substrate occurs. The swelling gelatinous

<sup>2</sup> Krawkow, S. Zhur. Obit. Agron., 9: 569-624. 1908; 10: 1-34. 1909.

<sup>3</sup> Kunze, F. Jahrb. wiss. Bot., 42: 357-391. 1906.

<sup>4</sup> Fischer, H. Centrbl. Bakt. II, 55: 1-5. 1921.

<sup>5</sup> Jensen, P. R. Intern. Rev. ges. Hydrob. Hydrogr. Leipzig, 2. 1909; Roux, M. Ann. biol. lacustre., 2. 1907.

<sup>6</sup> Vernadsky, W. J. Compt. Rend. Acad. Sci., 175: 450-452. 1922; Stoklasa, J. Über die Verbreitung des Aluminums in der Natur. G. Fischer, Jena. 1922; Thiel, G. A. Econ. Geol., 22: 480-493. 1927.

<sup>7</sup> Müntz, A. Compt. Rend. Acad. Sci., 110: 1370-1372. 1890; Merrill, G. P. Bul. Geol. Soc. Am., 6: 321-332. 1895.

apothecial or thallial tissue exerts a pull on the substrate, to which it is firmly attached; when the strain reaches the limit which either the lichen tissue or the substrate can sustain, a break in the one or the other occurs, the physical properties of the substrate determining the amount of mechanical disintegration which can take place in this way.<sup>8</sup> The lichens are soon followed by various algae, fungi and finally by bacteria. The action of these organisms is both mechanical and chemical; some organisms exert a predominating chemical and others a mechanical action.

Certain bacteria are capable of deriving their necessary mineral nutrients from feldspars, bringing considerable quantities of undecomposed orthoclase into solution, probably by means of the carbon dioxide which is formed.<sup>9</sup> The action of *B. extorquens*, nitrate-forming and butyric acid organisms, as well as yeasts, upon twelve different silicates and upon apatite was investigated. The bacteria were able, by means of their products of respiration, to dissolve considerable amounts of pulverized silicates; the formation of organic acids by *Bac. amylobacter* markedly influenced the solubility of silicates. The intensity of contact of the organism with the mineral to be acted upon was found to be of even greater importance than the other agents of solubility. Thus, *B. extorquens*, which produced only carbon dioxide but which formed a close and firm envelope around the mineral particles, had the strongest solvent action. Yeasts which did not form as close a contact, although they produced more carbon dioxide, brought about less solubility. Nitrite forming bacteria, as a result of the production of a strong inorganic acid ( $\text{HNO}_2$ ), caused a considerable solubility of the silicates. Minerals rich in alkaline earths were most readily acted upon. Apatite dissolved only to a limited extent in carbonic acid and only those bacteria which produced organic acids brought about considerable solubility. The alkalies came into solution first, followed by the alkaline earths and iron; silicic acid and aluminum oxide came into solution last. Kawamura<sup>10</sup> described an organism, *Volcanothrix silicophila*, found in the volcanic material in Japan at an altitude of 6600 feet, which formed a zoogloal mass, the ash of which contained 8.873 per cent silica. The

<sup>8</sup> Jennie Fry, E. Ann. Bot., 163: 437-460. 1927; Smith, A. L. Lichens. Cambridge. 1921; Tobler, S. Biologie der Flechten. Berlin. 1926, p. 182; Diels, L. Ber. deut. bot. Gesell., 31. 1913; Bachmann, E. Ibid., 31. 1913; 34. 1916; Schroeter, C. Pflanzenleben der Alpen. 2d Ed. Zürich. 1926, p. 756.

<sup>9</sup> Bassalik, K. Ztschr. Gärungsphysiol., 2: 1-32. 1912; 3: 15-42. 1913.

<sup>10</sup> Kawamura, T. (cited by Wright, p.

presence of bacteria was also found to increase the etching power of the roots of plants.<sup>11</sup>

A certain relationship exists between the hydrogen-ions produced by bacteria and the amount of bases brought into solution from the mineral, since the magnitude of the effect of bacterial end-products upon a mineral depends upon the equilibrium established.<sup>12</sup> It was suggested that the action of bacterial end-products, acid in nature, upon minerals is explainable as a chemical reaction. Fig. 45 shows the

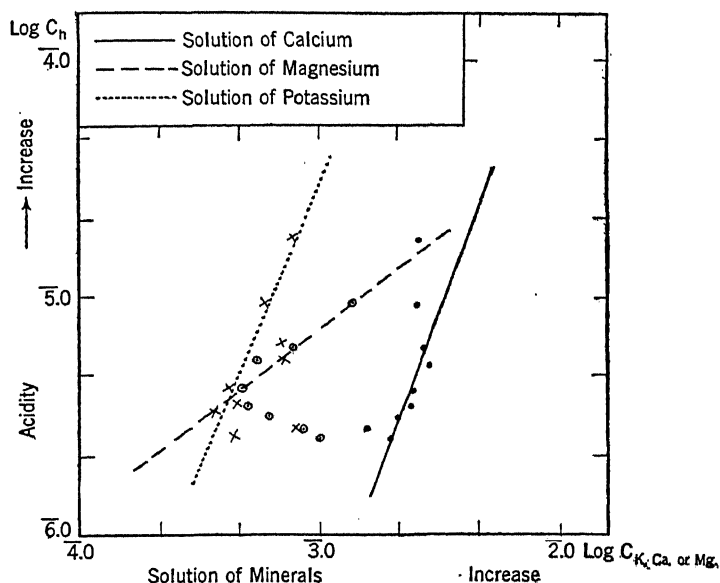


FIG. 45. The influence of acidity created by growth of *Azotobacter* on solution of calcium, magnesium and potassium from the mineral biotite (after Wright).

action of bacterial cultures upon a typical silicate. Various animals, including earthworms (to which attention was called by Darwin), ants, snails, play an active part in the disintegration of rocks and in soil formation.<sup>13</sup>

Definite information is available on the transformation of a few of the more important mineral elements by microorganisms; some of the rarer

<sup>11</sup> Fred, E. B. and Haas, A. R. C. *Jour. Gen. Physiol.*, 1: 631-638. 1919.

<sup>12</sup> Wright, D. *Univ. Cal. Publ. Agr. Sci.*, 4: 247-337. 1922.

<sup>13</sup> Shaler, N. S. *U. S. Geol. Survey*, 4: 213-345. 1892; Shimek, B. *Ecology*, 11: 673-686. 1930; Andrée, K. In *Salomon's Grundzüge der Geologie*, p. 726.

elements, such as Ni and Co, exist in the soil in small quantities and probably play a rôle in the activities of some of the microorganisms.

*Nature of phosphorus compounds in the soil.* Phosphorus undergoes various changes in the soil as a result of the activities of the soil microflora and microfauna. When organic matter is mineralized, the phosphorus is liberated in the form of inorganic salts; the latter may be re-assimilated by the same or by other microorganisms and synthesized into microbial protoplasm,<sup>14</sup> especially in the presence of available energy. Insoluble inorganic phosphates may be made soluble, as a result of the action of the products of microbial metabolism including carbon dioxide and the various organic and inorganic acids. The reduction of phosphorus compounds by bacteria has been brought out elsewhere (p. 490).

Phosphorus is present in normal soils in the form of inorganic and organic compounds. The inorganic compounds include mono-, di-, tri-, and tetra-phosphates of potassium, sodium, calcium, magnesium, aluminum, iron and manganese. The organic compounds comprise the phosphorus of plant and animal residues and that in the living or dead protoplasm of microorganisms: these include various compounds, namely nucleic acids, lecithin and phytin.<sup>15</sup> Phosphorus is usually added to the soil in the form of superphosphates, insoluble tri- and tetra-calcium phosphates, and organic forms, both in plant residues and in organic fertilizers. When soluble phosphates are added to the soil, they interact with the hydroxides, carbonates, silicates and tri-phosphates of calcium, magnesium, iron and aluminum to give insoluble precipitated phosphates. Superphosphates were found to change, in the presence of sufficient  $\text{CaCO}_3$  in the soil, into insoluble phosphates, within 50 to 60 days. Normal soils contain 0.025 to 0.3 per cent of phosphorus, very little of which is soluble in pure water, but quite appreciable quantities are soluble in water containing carbon dioxide. In fertile soils, the phosphorus is present partly in the form of organic phosphorus compounds. A part or even all the organic phosphorus found in the soil

<sup>14</sup> Fixation of phosphorus in soil is discussed in detail by Gaarder, T. Meddel. 14, Vestlands forstl. Forsok. Bergen. 1930; Barbier, A. G. Ann. Sci. Agron., 47: 329-363. 1930; Balanescu, G. Ibid. 364-375; Bytchinine, A. A. Ibid. 376-383.

<sup>15</sup> Shorey, E. Science N. S., 35: 390. 1911; Biochem. Bull., 1: 104. 1911; U. S. Dept. Agr., Bur. Soils, Bul. 88. 1913; Stoklasa, J. Centrbl. Bakt. II, 29: 385-519. 1911; Auten, J. T. Soil Sci., 16: 281-294. 1923; Aso, K. Bul. Coll. Agr. Tokyo Imp. Univ., 6: 277-294. 1904; see also Soil Sci., 2: 291. 1916; 13: 119-124. 1922; Ill. Agr. Exp. Sta. Bul., 145. 1910; Texas Agr. Exp. Sta. Bul., 136. 1911.

may be in the form of bodies of microorganisms.<sup>16</sup> The total phosphorus brought into the soil by a two ton crop of green manure (including roots) may amount to 20 to 50 pounds  $P_2O_5$  per acre. The  $P_2O_5$  content of straw is 0.15 to 0.30 per cent, of clover and timothy hay 0.50 to 0.55 per cent, of fresh horse manure (without straw) 0.34, cow manure 0.21, sheep manure 0.40, chicken manure 0.83 per cent.

The analysis of the dry matter of a few typical bacteria reported by Stoklasa shows that these organisms can store away considerable quantities of phosphorus (table 62). Seventy-nine to 81 per cent of this phosphorus was found to be in the form of nucleic acid and 7.6 to 8.6 per cent as lecithin. The ash of yeasts may consist of 60 per cent  $P_2O_5$ . The ash of fungi, however, contains a lower concentration of phosphorus than yeasts and bacteria, depending largely on the phosphoric acid content of the medium. A more or less constant nitrogen-phosphorus ratio ( $N:P_2O_5 = 4.2\%:2.0\%$ ) was found<sup>17</sup> in the dry mycelium of *Asp.*

TABLE 62

*Phosphorus and potassium content of some typical soil bacteria*

	Ash	TOTAL $P_2O_5$	TOTAL $K_2O$
	per cent	per cent	per cent
<i>Az. chroococcum</i> .....	8.2-8.6	4.93-5.2	2.41-2.65
<i>Bac. mycoides</i> .....	7.5	4.07	2.27
<i>Bact. fluorescens liquefaciens</i> .....	6.48	5.32	0.83

*niger*. A similar ratio is found in the cells of other organisms and in the soil organic matter, pointing to a definite C/P (organic) ratio in the soil.

*Decomposition of organic phosphorus compounds by microorganisms.* A large number of microorganisms including various heterotrophic bacteria, fungi and actinomyces, are capable of decomposing organic phosphorus compounds.

Lecithin contains 9.39 per cent  $P_2O_5$ , 1.6 per cent N and 65.36 per cent C. It contains two fatty acid radicals, usually palmitic and stearic or oleic, which are rather poor sources of carbon for microorganisms. In the presence of available carbon and nitrogen sources, microorganisms break down lecithin and liberate the phosphorus. Two-thirds of the lecithin phosphorus was transformed into soluble phosphate by different

<sup>16</sup> Gortner, R. A. and Shaw, W. M. Soil Sci., 3: 99-111. 1916.

<sup>17</sup> Schmücke, R. Biochem. Ztschr., 153: 372-423. 1924.

bacteria in 60 days, at 28° to 30°C; the rest of the phosphorus was re-assimilated by the organisms..

Phytin is an hexaphosphate, occurring abundantly in vegetable tissues, especially in seeds or grains.<sup>18</sup> It contains about 26 per cent phosphorus as phytic acid ( $C_6H_{24}O_{27}P_6$ ). It is acted upon by fungi and bacteria by means of an enzyme, phytase,<sup>19</sup> with the transformation of the phosphorus into the inorganic form.

Nucleo-proteins contain 7 to 9 per cent phosphorus and 13 to 14 per cent nitrogen. The resulting products of their decomposition are phosphoric acid, a sugar, purine and pyrimidine bases.<sup>20</sup> The nucleic acids are broken down by various soil microorganisms, both in the presence and absence of other nitrogen sources. In addition to some common soil organisms, a special group of bacteria (genus *Nucleobacter*) was found<sup>21</sup> to be specifically concerned in the decomposition of nucleins, through the nucleic acid stage, into phosphoric acid.

In addition to these compounds, other organic phosphorus compounds, such as that of wheat bran (inosite mono-phosphate— $C_6H_{13}O_8P$ ) are added to the soil, in considerable quantities. Their decomposition is probably similar to that of phytin.

*Transformation of insoluble tri-calcium phosphates into soluble forms by microorganisms.* Insoluble tri-calcium phosphates may be brought into solution by microbial action in three different ways: (1) by the direct metabolism of microorganisms, perhaps through the formation of some enzyme or by interaction with some synthesized substance, (2) by the action of carbon dioxide as well as various organic acids produced by soil organisms, (3) by the action of inorganic acids formed in the metabolism of the autotrophic nitrifying and sulfur oxidizing bacteria.

Soil microorganisms were found capable of rendering insoluble tri-calcium phosphate soluble when it is present in culture media as the only source of phosphorus. *Asp. niger*, *Pen. brevicaulis*, *Pen. glaucum* assimilated, in 60 days at 22°C., one-fifth to one-third of the phosphate

<sup>18</sup> Anderson, R. J. N. Y. (Geneva) Agr. Exp. Sta. Tech. Bul., 19, 1912; Bul. 25, 1912.

<sup>19</sup> Dox, A. W. and Golden, R. Jour. Biol. Chem., 10: 183-186. 1911; Plimmer, R. H. A. Biochem. Jour., 7: 43-71. 1913; Egorov, M. A. Ztschr. physiol. Chem., 82: 231-242. 1912.

<sup>20</sup> Ivanov, N. Ztschr. Gärungsphysiol. 1: 60. 1912; Ztschr. physiol. Chem., 39: 31-43. 1903; Schittenhelm, A. and Schroeter, F. Ztschr. physiol. Chem., 39: 203-207. 1903; 40: 62-69. 1903; 41: 283-292. 1903; 57: 21-27. 1908.

<sup>21</sup> Koch, A. and Oelsner, A. Biochem. Ztschr., 134: 76-96. 1922.

present as  $\text{Ca}_3(\text{PO}_4)_2$  in liquid culture media.<sup>22</sup> Out of twenty-five bacteria isolated from the soil, twelve had a definite solvent action on rock phosphate, bone, pure tri-calcium phosphate, di-calcium phosphate and calcium carbonate, when supplied with some form of sugar in the nutrient medium.<sup>23</sup> Both the acid formed by the bacteria and the carbon dioxide were found to be factors exerting the solvent action. The solubility of phosphates is influenced by the nature of the carbon and nitrogen sources for the growth of microorganisms;<sup>24</sup> disaccharides are better sources of carbon than monosaccharides and ammonium salts are better sources of nitrogen than other nitrogenous substances. The soluble bases, especially calcium, prevent the solubility of phosphates; iron oxide influences the process least. This would seem to indicate definitely that the solubilization of phosphate is a result of secondary reactions, depending upon the action of the products of carbon and nitrogen metabolism upon the insoluble phosphate.

The production of soluble phosphates was, however, also reported<sup>25</sup> to be associated with the vital functions of microorganisms. Bazarevski<sup>26</sup> questioned the existence of specific enzymes capable of bringing into solution insoluble phosphates and submitted evidence to show that this process is chiefly a result of acid production by microorganisms. Even if the organism does not form any acid in the medium, a part of the phosphate may be assimilated by it, either by the action of carbon dioxide or as a result of base exchange with the soluble products of microbiological reactions and certain soil constituents. The continuous removal of the soluble phosphate by the growing organism and its synthesis into organic matter may sometimes bring about appreciable transformation. The greatest amount of solubilization of the phosphate has been obtained with ammonium salts and the least with nitrate; this again substantiates the idea that the acids formed in the metabolism of the organisms, rather than any enzymatic phenomena, are responsible for the process. In the case of ammonium salts, the organisms remove the ammonium as a source of nitrogen, leaving the  $\text{SO}_4^{=}$  in the medium,

<sup>22</sup> de Grazia, S. and Gerza, U. *Ann. R. Sta. Chem. Agr. Sper. Roma* (II), **2**, 1908; **3**, 1909.

<sup>23</sup> Sackett, W. G., Patten, A. J. and Brown, C. W. *Centrbl. Bakt.* II, **20**: 688-703. 1908.

<sup>24</sup> Perotti, R. *Centrbl. Bakt.* II, **25**: 409-419. 1909.

<sup>25</sup> Pozerski, E. and Levy, M. M. *Compt. Rend. Soc. Biol.*, **87**: 1157-1159. 1922.

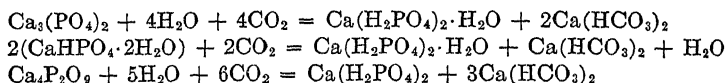
<sup>26</sup> Bazarevski, S. On the question of mobilization of phosphoric acid in the soil by the agency of microorganisms (Russian). *Moskau*. 1916.



while in the case of the nitrate, the acid-ion is removed and the base is left.<sup>27</sup>

The growing organism is capable of forming large quantities of carbon dioxide and often appreciable quantities of organic acids, both under aerobic and anaerobic conditions. One gram of bacterial cells produces 0.25 to 0.5 mgm. of CO<sub>2</sub> in twenty-four hours and 1 gram of fungus mycelium produces 0.13 to 0.18 mgm. CO<sub>2</sub>. As much as 6000 pounds of CO<sub>2</sub> may be given off in 200 days by one acre of normal soil, on a 2,000,000 pounds basis. The soil atmosphere may contain 0.6 to 3.8 per cent of CO<sub>2</sub>. Czapek<sup>28</sup> ascribed to carbon dioxide the most important rôle in bringing the soil minerals into solution.

Carbon dioxide interacts with the different phosphates in the following manner:



One kilogram of soil containing 0.13 per cent P<sub>2</sub>O<sub>5</sub>, when extracted for twenty-five days with distilled water (five repeated extractions of five days each), yielded 0.002 gram P<sub>2</sub>O<sub>5</sub>, while 0.0085 grams P<sub>2</sub>O<sub>5</sub> was extracted in the same period by water containing carbon dioxide.<sup>29</sup>

The various organic acids (butyric, lactic, acetic, citric, oxalic, fumaric, etc.) formed by soil organisms interact with the phosphates, carbonates and silicates present in the soil, to give lactates, acetates, butyrates, citrates, etc. These are usually further decomposed by different microorganisms to carbon dioxide and carbonates. Table 63 shows the amounts of various phosphates made soluble when extracted by dilute solutions of organic acids (for 500 hours), by water saturated with carbon dioxide and by the growth of *Azotobacter chroococcum*, the particular phosphate being the only source of phosphorus in the medium.

In the case of bone meal, the solubility of the phosphate was found to depend on the fineness of division, amount and composition of the fat and nitrogen in the keratin and collagen. The decomposition of organic matter in soil is thus found to influence to a large extent the process of rendering insoluble soil phosphorus available.<sup>30</sup>

<sup>27</sup> Haselhoff, E. Landw. Vers. Sta., 70: 53-143. 1909; Stålström, A. Centrbl. Bakt. II, 11: 724-732. 1904.

<sup>28</sup> Czapek, 1920-1921 (p. XVII).

<sup>29</sup> Stoklasa, 1911 (p. 570).

<sup>30</sup> Kröber, E. Jour. Landw., 57: 5-80. 1909; Koch, A. and Kröber, E. Fühlings landw. Ztg., 55: 225-235. 1906.

The activities of soil microorganisms do not always result in an increase of soluble phosphates, but may often lead to an actual diminution.<sup>31</sup> This takes place especially when a large amount of energy bearing materials is added to the soil without a corresponding addition of soluble phosphates. The available energy stimulates the activities of various soil microorganisms, the synthesizing processes of which result in a disappearance of the soluble phosphates in the soil. The actual amount of available phosphorus in soil is a result of the sum total of the activities of the microbial soil population which includes acid formation, secondary reactions and the synthesizing activities.

TABLE 63  
*Solubility of different phosphates*

PHOSPHATE	P <sub>2</sub> O <sub>5</sub> CONTENT	SOLUBLE IN 0.5 PER CENT ACETIC ACID	SOLUBLE IN 0.5 PER CENT FORMIC ACID	SOLUBLE IN CO <sub>2</sub> WATER	DISSOLVED AND ASSIMI- LATED BY AZ. CHROO- COCCUM
	per cent	per cent	per cent	per cent	per cent
Di-calcium.....	41.0	97.13	99.54	45.79	32.41
Tri-calcium.....	41.0	73.83	90.90	25.01	24.89
Mono-diferro.....	47.0	19.58	29.35	27.44	40.94
Tri-ferri.....	38.0	8.13	16.00	7.87	9.05
Tri-aluminum.....	44.0	22.09	93.79		10.71
Florida rock.....	36.0	16.31	54.04		18.57
Steamed bone meal.....	21.0	62.25	67.65		
Dry granitic soil.....	0.103	7.76	6.79	4.85	49.02
Dry basalt soil.....	0.180	7.22	8.33	5.50	50.27

This is brought out in table 64. Soil was sterilized in the autoclave and reinoculated with pure cultures of bacteria or with soil suspensions, and, after a certain period of incubation, the amounts of carbon dioxide formed from 1,100 gm. of soil and the phosphorus brought into solution were determined.<sup>31</sup> On inoculating sterilized soil with pure cultures of bacteria, Sewerin obtained a gain of 14 per cent of P<sub>2</sub>O<sub>5</sub> soluble in acetic acid for *Azotobacter* + *Bacterium* sp.; a gain of 12.9 per cent for *Bac. mesentericus vulgatus*; a gain of 8.0 per cent for *Bact. radicola* + *Azotobacter*; a loss of 5.8 per cent for *Bact. fluorescens liquefaciens*; and a loss of 12.6 per cent for unsterilized soil. No correlation was found between the bacterial population of the soil and the soluble P<sub>2</sub>O<sub>5</sub>, and none between the latter and the energy of decomposition of the soil organic matter.

<sup>31</sup> Sewerin, S. A. Centrbl. Bakt. II, 28: 561-580. 1910; 32: 498-520. 1912; Viestnik Bakteriolo.-Agron. Stan. 21: 53-83. 1914.

When manure is added to the soil, the rapidly growing bacteria cause a definite decrease in the water-soluble phosphorus of the manure, and transform it into organic phosphorus. This is eventually released in an available form as a result of the action of bacteria on the dead microbial cells, after the available energy had been used up.<sup>32</sup> The addition of green manure and stable manure to citrus soils in California was found to bring about a measurable increase in solubility of phosphorus, calcium, magnesium and iron.<sup>33</sup>

The addition of carbohydrates to the soil brings about an increase in the number of microorganisms and a diminution in the amount of phosphoric acid soluble in 2 per cent acetic acid.<sup>34</sup> The amount of soluble phosphate in the soil depends not so much upon the numbers of

TABLE 64

*Influence of soil sterilization upon the activities of microorganisms and transformation of phosphorus*

SOIL TREATMENT	CO <sub>2</sub> FORMATION		NITRATE FORMATION IN 100 GRAMS OF SOIL	SOLUBLE PHOSPHATE	NUMBERS OF BACTERIA PER GRAM
	30 days	60 days			
	mgm.		mgm. of N <sub>2</sub> O <sub>5</sub>	per cent P <sub>2</sub> O <sub>5</sub>	millions
Sterile soil.....	0.281	0.418	48.3	0.0060	
Sterile soil inoculated with fresh soil suspension.....	7.636	10.854	114.0	0.0040	22.8-26.7
Non-sterilized soil.....	2.962	4.640	12.3	0.0014* 0.0018	3.2- 5.8

\* Upper figure found at beginning, lower at end of experiment.

microorganisms as upon their kind. As to the influence of season of year, an increase in soluble phosphate is usually found in the spring and fall and a decrease in the summer, as a result of the activities of microorganisms. The amount of available phosphorus in soil will thus depend on the total phosphorus in the soil, nature of the phosphorus compounds, soil reaction, presence of available energy and nitrogen, and kind of microorganisms.

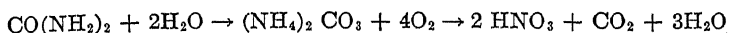
*Transformation of insoluble phosphates by inorganic and organic acids formed by microorganisms.* The continued oxidation in normal soils of ammonium salts to nitrous and nitric acids results in the formation

<sup>32</sup> Tottingham, W. E. and Hoffmann, C. Wis. Agr. Exp. Sta. Res. Bul., 29: 273-321. 1913.

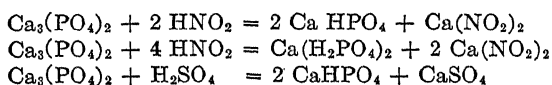
<sup>33</sup> Jensen, C. A. Jour. Agr. Res., 9: 253-268. 1917.

<sup>34</sup> Bazarevski, 1916 (p. 573).

of appreciable quantities of these acids, 132 parts of ammonium sulfate giving, on complete oxidation, 126 parts of nitric and 196 parts of sulfuric acids. Even organic compounds rich in nitrogen, like urea, finally lead to a high acidity.



The acids may, under proper conditions of reaction, interact with the tri-calcium phosphate added to the soil and make it soluble.



Theoretically 188 parts of nitrous acid mixed with 310 parts of pure rock phosphate should give 234 parts of acid phosphate and 264 parts of calcium nitrite. As the average of thirteen tests in liquid culture, Hopkins and Whiting<sup>35</sup> found that 115 parts of phosphorus and 211 parts of calcium were made water soluble for every 56 parts of nitrogen oxidized by the nitrite forming bacteria. No further increase was obtained from the action of the nitrate bacteria, since the oxidation of the nitrite to nitrate does not bring about any further increase in acidity.

However, the reactions taking place in the soil are not similar to those observed in liquid cultures, due to the fact that the nitrous acid combines in the soil with calcium and magnesium carbonate and salts of organic acids liberating rather weak organic acids, as shown in table 65.<sup>36</sup> The acids resulting from the activities of the bacteria neutralize the carbonate in preference to the tri-calcium phosphate in the soil. When considerable acidity is produced, as in the presence of ammonium sulfate, some phosphate goes into solution. But the degree of acidity necessary for the transformation of the tri-calcium phosphate into soluble phosphates is so high (p. 580) that it may become distinctly injurious to crop growth. The amount of phosphate that goes into solution, as a result of the activities of the nitrifying bacteria is, therefore, very limited. Nitrification of organic nitrogenous compounds like dried blood does not increase the amount of citrate-soluble phosphate when rock phosphate is added to the soil. The nitrification of ammonium sulfate in normal soils does not have any appreciable effect upon the solubility of rock phosphate. However, the concentration of the water-soluble calcium is increased in both instances, due largely to the

<sup>35</sup> Hopkins, C. G. and Whiting, A. L. Ill. Agr. Exp. Sta. Bul. 190. 1916.

<sup>36</sup> Kelley, W. P. Jour. Agr. Res., 12: 671-683. 1918.

action of the acid upon the calcium present in the soil in the form of silicates.<sup>37</sup>

The transformation of rock phosphate into soluble forms by sulfuric acid formed from the oxidation of sulfur by microorganisms is very similar to its transformation by the nitrous acid. In pure culture or in composts, the transformation of the phosphate is rapid and almost complete. In the soil, the sulfuric acid tends to transform the calcium

TABLE 65

*Effect of nitrification on the solubility of tricalcium phosphate in soil\**

MATERIALS ADDED	AFTER 28 DAYS			AFTER 57 DAYS			AFTER 157 DAYS		
	NO <sub>2</sub> -N	Ca	P <sub>2</sub> O <sub>5</sub>	NO <sub>2</sub> -N	Ca	P <sub>2</sub> O <sub>5</sub>	NO <sub>2</sub> -N	Ca	P <sub>2</sub> O <sub>5</sub>
	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.
Control.....	20.0	45.0	13.1	25.5	50.6	11.0	.....	.....	.....
CaCO <sub>3</sub> .....	22.0	56.5	11.9	29.0	70.8	13.2	.....	.....	.....
Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> .....	21.0	53.5	24.2	28.0	58.8	25.0	.....	.....	.....
CaCO <sub>3</sub> + Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> .....	22.0	59.1	17.3	28.0	70.1	22.4	.....	.....	.....
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .....	98.0	219.4	18.5	99.0	225.4	19.4	114.0	232.1	8.0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> + CaCO <sub>3</sub> .....	97.0	254.4	18.5	98.0	270.5	7.4	.....	.....	.....
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> + Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> .....	99.0	217.7	52.1	99.0	229.6	38.0	111.0	218.4	30.0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> + CaCO <sub>3</sub> + Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> .....	100.0	253.4	26.6	101.0	230.4	13.9	.....	.....	.....
Dried blood.....	91.0	107.7	9.7	90.0	113.9	10.0	94.0	116.4	5.7
Dried blood + CaCO <sub>3</sub> .....	89.0	107.2	9.8	90.0	140.2	11.5	.....	.....	.....
Dried blood + Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> .....	82.0	111.7	24.3	88.0	117.7	22.2	.....	.....	.....
Dried blood + CaCO <sub>3</sub> Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> .....	81.0	118.2	19.5	87.5	138.1	18.3	.....	.....	.....

\* The ammonium sulfate was added at the rate of 0.01 gram of nitrogen per 100 grams of soil; an equal quantity of nitrogen was added in the form of dried blood; tri-calcium phosphate—0.10 gram and calcium carbonate—0.25 gram per 100 grams of soil.

and magnesium carbonates, the silicates and salts of organic acids, in preference to the phosphate. The reaction of the medium has to be distinctly acid (pH 3.0) for the phosphate to become soluble. Such acidity is injurious to plant growth. Figure 46 shows the correlation between soil reaction and transformation of tri-calcium phosphate.<sup>38</sup> When rock phosphate is used in place of tri-calcium phosphate, the

<sup>37</sup> Ames, J. W. Ohio. Agr. Exp. Sta. Bul. 351. 1921.

<sup>38</sup> Rudolfs, W. Soil Sci., 14: 247-262. 1922.

reactions involved are more complicated, since it contains aluminum and iron phosphates in addition to calcium phosphate, as shown by the following analysis of Florida rock:

	<i>per cent</i>		<i>per cent</i>
P <sub>2</sub> O <sub>5</sub> .....	35.52	Al <sub>2</sub> O <sub>3</sub> .....	1.03
CaO.....	49.92	SiO <sub>2</sub> .....	3.57
MgO.....	0.46	CO <sub>2</sub> .....	1.64
Fe <sub>2</sub> O <sub>3</sub> .....	0.98	SO <sub>3</sub> .....	0.21
		H <sub>2</sub> O.....	2.67

The reactions involved in the conversion of the rock phosphate into soluble forms (di- and monocalcium phosphate and phosphoric acid) by means of acid belongs to the type of reactions of heterogenous systems. The rock phosphate minerals have no definite composition and the products formed are not always the same. The following factors control the reaction:<sup>39</sup> (1) concentration of the reacting mass; (2) temperature of the reacting medium; (3) the amount of contact of the reacting substances; (4) the speed of diffusion of the reacting substances; and (5) catalytic agents. In addition to these, other factors are of importance, including the chemical composition and the physical properties of the solid phase. These have a tremendous influence on the speed of the reaction and they are the least known, since the chemical makeup of the rock phosphate is still obscure.

The nature of the phosphate influences considerably the liberation of phosphate ions as a result of soil acidity. Iron phosphate is least soluble at pH 3.0; when the reaction is less acid, ferric hydroxide is precipitated at the expense of ferric phosphate and phosphate ions are liberated. Calcium phosphate is insoluble under alkaline conditions; an excess of calcium ion in the presence of hydroxyl ion depresses the solubility of calcium phosphate; the removal of the calcium ion causes an increase in the liberation of phosphate ion. Manganese and aluminum phosphates are least soluble under slightly acid conditions; under alkaline conditions, manganic oxide and aluminate ions liberate phosphate ions.<sup>40</sup>

Schloesing<sup>41</sup> showed in 1898 that the quantity of phosphoric acid dissolved in normal soils is a result of an equilibrium of very complex chemical processes tending, on the one hand, to take this acid out of

<sup>39</sup> Kazakov, A. V. Moskau Inst. Agron., 9: 21-45, 57-68. 1913.

<sup>40</sup> Teakle, L. J. H. Soil Sci., 25: 143-162. 1928.

<sup>41</sup> Schloesing fils, Th. Compt. Rend. Acad. Sci., July 25, 1898.

solution and, on the other, to bring it into solution. It may only be added that these chemical processes are brought about by the activities of microorganisms, and whatever will influence these activities will also influence the amount of phosphorus available in the soil at any given time.

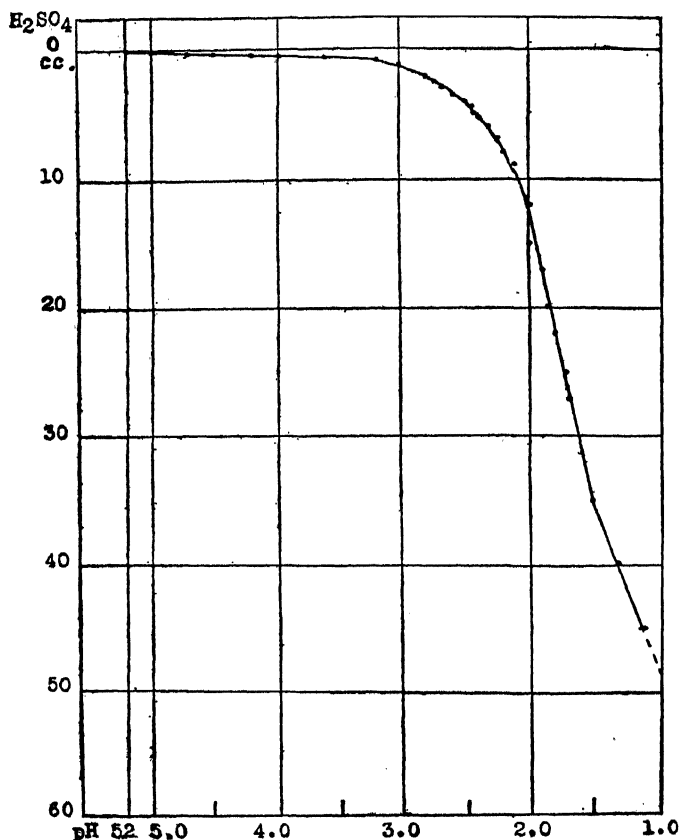
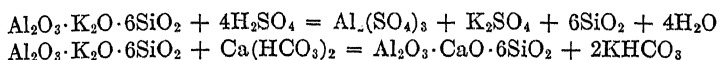


FIG. 46. Hydrogen-ion concentration at which rock phosphate becomes available (from Rudolfs).

*Transformation of potassium in the soil by microorganisms.* Potassium is present in soil in the form of organic compounds and various zeolitic and non-zeolitic silicates. The potassium added to the soil is either in a soluble inorganic form, an insoluble inorganic form (marl), or an insoluble organic form (manures). The  $K_2O$  content of straw,

hay and grain ranges from 0.5 to 2.0 per cent and from 0.288 to 0.504 per cent in the case of fresh manure.<sup>42</sup> The ash content of bacterial cells contains 4.0 to 25.6 per cent  $K_2O$  and of fungus mycelium 8.7 to 39.5 per cent. The activities of bacteria may lead to an increase in the available potassium, as in the decomposition of organic matter by microorganisms and in the formation of acids which liberate potassium from zeolites. Orthoclase, for example, may interact with acids formed by microorganisms or with the calcium bicarbonate formed from insoluble calcium phosphate by the action of carbon dioxide, to give, in both cases, soluble potassium salts:



The process of replacement of basic ions in the zeolitic part of the soil is of common occurrence, the hydrogen ion acting also as a base. The solubility of the potassium, often ascribed to the action of acids, may be due merely to the replacement of the potassium in the zeolitic complexes by the calcium or magnesium salts of organic or inorganic acids added to the soil. However, when feldspar, glauconite or other silicates rich in potassium are composted with substances which result in the formation of acid (e.g., sulfur), a great deal of the potassium may go into solution (p. 540).

By composting greensand, sulfur, manure and soil, 9.1 to 41.3 per cent of the total initial potassium present can be made soluble.<sup>43</sup> The results of Wright cited previously on the action of organic acids upon silicates containing potassium tend to confirm this assumption. This process takes place only very slowly in normal soils. According to Ames,<sup>44</sup> the liberation of potassium in the soil is brought about by the salts formed rather than by the direct action of acidity on insoluble potassium compounds, although he found that both nitrification of organic and inorganic nitrogen compounds and oxidation of sulfur in the soil increased the amount of water-soluble potassium.

The available potassium compounds are also readily assimilated by the heterotrophic bacteria and fungi and are stored away in their mycelium;<sup>45</sup> when this is decomposed, the potassium again becomes

<sup>42</sup> Thorne, 1914 (p. 411); Bartholomew, 1928 (p. 630).

<sup>43</sup> McCall, A. G. and Smith, A. M. Jour. Agr. Res., 19: 239-256. 1920; Jour. Assoc. Offic. Agr. Chem., 5: 133-136. 1921.

<sup>44</sup> Ames, 1921 (p. 578).

<sup>45</sup> Kyropoulos. Ztschr. Gärungsphysiol., 5: 161. 1915.



available. Only a part of the free potassium salt remains in the soil as such; it also replaces some of the zeolitic bases, such as Ca, Mg, Na. The available potassium in the soil at any given time depends not only on the total content of this element, but also on the form in which it is present in the soil, the degree of saturation of zeolitic compounds, soil reaction, available organic matter and activities of various groups of microorganisms.

It has been suggested<sup>46</sup> to determine the available potassium in soil by inoculating a sterile nutrient solution containing a definite amount of soil as the only source of potassium with *Azotobacter*, incubating and determining the amount of nitrogen fixed. In the case of a fertile soil with a total of 0.093 per cent  $K_2O$ , 27.4 per cent was found to be available; in the case of a soil of medium fertility with a total of 0.27 per cent  $K_2O$ , only 5.46 per cent was available; in the case of a poor forest soil with a total of 0.137 per cent  $K_2O$ , only 2.18 per cent was available.

*Transformation of calcium and magnesium in soil.* In normal soils, calcium forms the chief base with which the soil zeolites and organic complexes are saturated. If an alkali salt is added to the soil, the base is absorbed and an equivalent amount of calcium is replaced. The activities of the microorganisms affect the transformation of calcium in the soil in various ways. (1) Calcium salts, particularly calcium carbonate, may be precipitated,<sup>47</sup> as a result of the interaction of the soluble calcium salts (of organic acids or nitrates) with carbonic acid produced by the decomposition of organic matter. (2) Calcium carbonate may be made soluble, as a result of activities of microorganisms, in a manner similar to that of the phosphates. The calcium is made soluble much more easily than the phosphate, since organic and inorganic acids interact very readily with calcium carbonate, as well as with calcium silicate in the soil, with the formation of soluble calcium compounds.

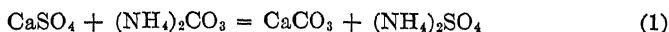
Nadson<sup>48</sup> found that soil bacteria may bring about the formation of

<sup>46</sup> Stoklasa, 1925 (p. 545).

<sup>47</sup> Gimingham, C. T. Jour. Agr. Sci., 4: 145-149. 1911; Drew, G. H. Carnegie Inst. Washington, Dept. Marine Biology, Papers from Tortugas Labor, 5: 7-45. 1914; Kellermann, K. F., and Smith, N. R. Jour. Wash. Acad. Sci., 4: 400-402. 1914; Naslund, C. Biochem. Ztschr., 184: 1-9. 1927; Nadson, G. A. Arch. Hydrobiol., 19: 154-164. 1928.

<sup>48</sup> Nadson, G. Mikroorganizmi kak geologitscheskie dieiateli. St. Petersburg. 1903.

calcium carbonate by means of the ammonium carbonate which is produced in the decomposition of organic matter:



The formation of calcium carbonate was also observed in the decomposition of organic compounds containing calcium. The mineral dolomite, a calcium and magnesium carbonate, was formed in media containing bacterial mixtures or a pure culture of *Bact. vulgare*. According to Molisch,<sup>49</sup> various bacteria, yeasts and actinomycetes are capable of causing the precipitation of calcium salts; but his use of the term "calcium bacteria" for these organisms is not justified.

Kellerman and Smith suggested that calcium carbonate precipitation takes place in any of the following ways: (1) Nitrates are reduced to nitrites and ammonia; the ammonia unites with  $\text{CO}_2$  to form  $(\text{NH}_4)_2\text{CO}_3$ , which reacts with  $\text{CaSO}_4$  to form  $\text{CaCO}_3$ . (2) Ammonia itself may act upon calcium bicarbonate and precipitate  $\text{CaCO}_3$ :



(3) The bacteria utilize organic acids as sources of energy; the calcium, with which the organic acids were combined in the form of salts, is thus liberated and reacts with the free  $\text{CO}_2$  to give precipitated  $\text{CaCO}_3$ . The calcium brought into solution by the organic and inorganic acids formed through the activities of microorganisms is either removed by the plant or is lost in drainage waters. The washing out of the calcium from the soil is sometimes so great that some soils of calcareous origin are practically free from lime.<sup>50</sup> The following reactions are involved in these processes:

1.  $\text{CaCO}_3 + (\text{NH}_4)_2\text{SO}_4 = \text{CaSO}_4 + (\text{NH}_4)_2\text{CO}_3$
2.  $(\text{NH}_4)_2\text{SO}_4 + 4\text{O}_2 = 2\text{HNO}_3 + \text{H}_2\text{SO}_4 + 2\text{H}_2\text{O}$   
 $\text{CaCO}_3 + \text{H}_2\text{SO}_4 = \text{CaSO}_4 + \text{H}_2\text{O} + \text{CO}_2$   
 $\text{CaCO}_3 + 2\text{HNO}_3 = \text{Ca}(\text{NO}_3)_2 + \text{H}_2\text{O} + \text{CO}_2$
3.  $\text{Ca}(\text{H}_2\text{PO}_4)_2 + \text{CaCO}_3 = 2\text{CaHPO}_4 + \text{H}_2\text{O} + \text{CO}_2$
4.  $\text{CaCO}_3 + \text{CO}_2 + \text{H}_2\text{O} = \text{Ca}(\text{HCO}_3)_2$

Applications of fertilizers, which are directly acid or which lead to the formation of acids in the soil, such as ammonium sulfate, superphosphate and sulfur, lead to a depletion of the calcium content of the soil.

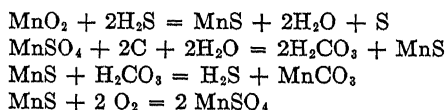
<sup>49</sup> Molisch, H. Centrbl. Bakt. II, 65: 130-139. 1925.

<sup>50</sup> Hall, A. D. and Miller, N. H. J. Proc. Roy. Soc. (London), B., 77: 1-32. 1905.

Calcium is also used directly as a nutrient by various microorganisms and small amounts of it may be assimilated. However, its chief value lies in the neutralization of organic and inorganic acids in the soil and in replacing injurious soil bases (sodium in alkaline soils), thus producing a medium more favorable for the growth of plants and microorganisms. Calcium salts also neutralize to some extent the injurious action of soluble magnesium salts, as shown by studies<sup>51</sup> on the influence of the lime-magnesia ratio upon crop growth. This ratio is not of great importance in bacterial activities, but the concentration of magnesium in solution and its relation to the concentration of the other constituents are of great importance.<sup>52</sup>

Magnesium is present abundantly in the soil (in the form of a zeolite saturating base) and is added to it in the form of various inorganic (dolomitic limestone, rock phosphate) and organic compounds. In the metabolism of bacteria and particularly fungi, magnesium may form a more important mineral nutrient than calcium. The transformation of magnesium in the soil is very similar to that of calcium, although Ames found that magnesium compounds are less resistant than calcium salts to the action of nitric and sulfuric acids formed in the processes of nitrification and sulfur oxidation.

*Transformation of manganese in soil.* Beijerinck<sup>53</sup> described several fungi and bacteria which are capable of oxidizing manganese carbonate to oxides of manganese, using cellulose or other carbohydrates as sources of energy. It has been further shown<sup>54</sup> that, in alkaline media, manganese salts are changed to manganese hydroxide, which is then oxidized by atmospheric oxygen to  $MnO_2$ . Microorganisms also oxidize manganese salts of organic acids to manganese carbonate. Manganic compounds may be reduced to manganous compounds by biological processes.



When sulfur is oxidized in the soil, considerable quantities of manganese may go into solution, especially in soils rich in this element. The

<sup>51</sup> Lemmermann, O., Einecke, A. und Fischer, H. Landw. Jahrb., 40: 173-254. 1911. Kelley, W. P. Centrbl. Bakt. II, 42: 519-526, 577-582. 1915.

<sup>52</sup> Greaves, J. E. and Carter, E. G. Soil Sci., 7: 121-160. 1919.

<sup>53</sup> Beijerinck, M. W. Folia Microb., 2: 123-135. 1913.

<sup>54</sup> Schöngen, N. L. Centrbl. Bakt. II, 40: 545-554. 1914.

utilization of certain manganese compounds as sources of energy by some of the iron bacteria has been pointed out elsewhere (p. 92). Small quantities of manganese seem to act as a stimulant for various organisms, especially the nitrogen-fixing bacteria.<sup>55</sup>

*Transformation of iron and zinc by microorganisms.* Various microorganisms are capable of extracting iron from solution and collecting it on their surfaces in the form of ferric hydroxide. However, only very few of these bacteria are capable of utilizing the energy liberated from the oxidation of the ferrous-ion to the ferric form for the chemosynthetic assimilation of carbon dioxide (p. 93). Iron has a very intimate relation with life in general and with the primitive forms of life in particular.<sup>56</sup> Here belong such processes as oxidation and hydration, which are of primary importance in the production of the colloidal condition of iron and which are fundamental in the maintenance of life.

The formation of ore deposits, especially iron and manganese ores<sup>57</sup> is frequently a result of the activities of microorganisms, such as the autotrophic iron bacteria, or of the modification of the environment by the development of heterotrophic organisms.<sup>58</sup> The accumulation of iron sulfides may be due to indirect microbial action, as in the precipitation of iron salts by hydrogen sulfide produced by the decomposition of proteins under anaerobic conditions, or reduction of sulfates; the same is true of the deposition of iron silicates.

Certain microorganisms are capable of oxidizing zinc sulfide (blende) to zinc sulfate. The transformation is favored by the presence of sulfur.<sup>59</sup> Sulfur oxidizing bacteria can bring about the dissolution of natural silicates and carbonates of zinc by the sulfuric acid formed from the oxidation of the sulfur.

*Transformation of aluminum in soil.* Aluminum forms one of the most abundant elements in soil, especially in clay soils, where it occurs in combination with silicates and with organic substances. It may even form definite compounds with some of the constituents of the soil

<sup>55</sup> Pietruszczynski, Z. Roczn. Nauk Rolnicz., 9: 235-287. 1923; Olaru, 1920 (p. 466).

<sup>56</sup> Francis, W. D. Proc. Roy. Soc. Queensland, 37: 98-107. 1925.

<sup>57</sup> Perfiliev, B. W. Verhandl. Intern. Vereinig. theoret. angew. Limnol., 37: 98-107. 1925.

<sup>58</sup> Harder, 1919 (p. 90); Starkey, R. L. and Halverson, H. O. Soil Sci., 24: 381-402. 1927.

<sup>59</sup> Rudolfs, W. and Hellbronner, A. Soil Sci., 14: 459-464. 1922; Compt. Rend. Acad. Sci., 174: 1373-1380. 1922; Dufrenoy, J. Ann. Soc. Hydrob. Med., 64: 26. 1922.

organic matter.<sup>60</sup> The transformation of organic and inorganic substances in the soil by microorganisms affects directly or indirectly the solubility and condition of aluminum in soil; the oxidation of sulfur brings into solution considerable quantities of aluminum; the same is true of the formation of nitrous and nitric acids from ammonia in soils deficient in bases.<sup>61</sup>

Aluminum may be liberated from hydrous aluminum silicates in the zone of rock decomposition, especially in the presence of small amounts of iron and sulfate waters, by microorganisms.<sup>62</sup> The very origin of kaolinite is believed<sup>63</sup> to be a result of bacterial action.

*Summary.* The various minerals studied here are more or less essential in the metabolism of microorganisms. Some organisms are capable of developing in media containing considerable concentrations of various salts.<sup>64</sup> Investigators frequently differentiate between fixed and free salts, in regard to their influence upon bacteria.<sup>65</sup> The presence of various minerals in soil is essential not only to the nutrition of microorganisms, but also for the purpose of neutralizing an unfavorable reaction, which results from the activities of various organisms. The soil bases, including iron and aluminum hydrates,<sup>66</sup> are especially important in this respect. The maintenance of a proper reaction in the soil is very essential for the activities of the nitrate-forming, nitrogen fixing and various cellulose decomposing bacteria.

The activities of microorganisms which result in a change of minerals from one chemical state into another may be classified as follows: (1) Heterotrophic energy utilization of microorganisms leads to a mineralization of the soil organic matter or to the liberation of minerals from their combination with organic compounds. (2) A part of the minerals thus liberated or added to the soil in the form of inorganic fertilizers may be reassimilated by various soil organisms and changed from a soluble into an insoluble condition. (3) The autotrophic bacteria,

<sup>60</sup> Niklas, 1912 (p. 566); Ostwald, W. and Steiner, A. *Kolloidchem. Beih.*, 21: 97-170. 1925.

<sup>61</sup> Further information on the transformation of aluminum in the soil is given by Stoklasa, 1922 (p. 567).

<sup>62</sup> Thiel, G. *Econ. Geol.*, 22: 480. 1927.

<sup>63</sup> Logan, W. N. *Kaolin in Indiana*. Pub. 6, Dept. Conserv. Indiana. 1919; see Bucher, W. H. *Econ. Geol.*, 16: 481. 1921.

<sup>64</sup> Sperlich, A. *Centrbl. Bakt.* II, 34: 406-430. 1912.

<sup>65</sup> Guillemin, M. and Larson, W. P. *Jour. Inf. Dis.*, 31: 349-355. 1922.

<sup>66</sup> Whiting, A. L. *Jour. Amer. Soc. Agron.*, 15: 277-289. 1923.

utilizing minerals as sources of energy, bring about a change in the chemical nature of the minerals in question. (4) The interaction between insoluble minerals in soil with the products formed by the activities of microorganisms, especially organic and inorganic acids, results in an increase in solubility of these minerals.

Various minerals, such as zinc, iron and copper, are quite essential, even if only in mere traces, for the activities of various organisms. Zinc stimulates the vegetative growth of *Asp. niger* and injures spore formation.<sup>67</sup> Copper is quite essential for the formation of the black pigment of *Asp. niger* and exerts a stimulating effect upon growth.<sup>68</sup> Arsenic also favors various microbial processes; the action of this mineral is probably of the nature of partial sterilization of soil.<sup>69</sup>

<sup>67</sup> Steinberg, R. A. Amer. Jour. Bot., 6: 330-372. 1919; Butkewitsch, W. and Orlow, W. G. Biochem. Ztschr., 132: 556-565. 1922.

<sup>68</sup> Roberg, M. Centrbl. Bakt. II, 74: 333-370. 1928; Bortels, H. Biochem. Ztschr., 182: 301-358. 1927.

<sup>69</sup> Greaves, J. E. Jour. Agr. Res., 6: 389-416. 1916; Soil Sci., 7: 121-160. 1919.

## CHAPTER XXIV

### MECHANISM OF DECOMPOSITION OF COMPLEX ORGANIC MATERIALS IN SOIL AND IN COMPOST BY MICROORGANISMS

Large quantities of organic matter are added constantly to the soil, in the form of plant stubble and other plant residues (leaves, needles, twigs and branches), green manures, stable manures, as well as organic fertilizers, both of plant and animal origin. This organic matter is immediately attacked by various groups of microorganisms, as soon as moisture and temperature conditions are favorable. The nature and rate of decomposition of the organic matter, as well as the formation of humus, or of soil organic matter, depend: (1) upon the chemical composition of the residues, which is controlled by the nature of the plant, its age, conditions of its nutrition, etc.; (2) upon the microorganisms active in the decomposition processes; (3) upon the environmental conditions under which decomposition is carried out, especially reaction, temperature, aeration and moisture supply; and (4) upon the presence of available inorganic nutrients, especially nitrogen, phosphorus and calcium.

*Chemical composition of plant materials.* The most important plant constituents may be classified into several groups, on the basis of their chemical composition: (1) sugars, starches, and other simple carbohydrates, most of which are soluble in cold or hot water; (2) pentosans, pectins, and other hemicelluloses, such as galactans and mannans, readily hydrolyzed by dilute acids; (3) true cellulose; (4) lignin and tannins; (5) fats, waxes, oils, sterols, and fatty acids; (6) proteins and their derivatives; (7) mineral constituents. Marked differences exist between these groups of complexes based on the ease of their decomposition in soil and the types of organisms capable of attacking them. A study of the decomposition of plant residues may deal with the action of microorganisms upon one particular chemical constituent, whereby unmodified plant material or chemically pure substances are employed, or with the plant substance as a whole. In this connection, it is of importance to know the chemical composition of the plant material, so as to be able to evaluate the processes of decomposition taking place. For this purpose

several systems of analysis have been proposed, which are all proximate, i.e., they do not attempt to account for 100 per cent of the plant material but only for the most important groups of constituents, which have been mentioned above.

To proceed with the chemical analysis, the plant material is first air dried, and separate samples (1 to 5 gm.) taken for determinations of moisture, ash by ignition, and total nitrogen by the Kjeldahl method. When the organic material has already undergone a certain amount of decomposition, and frequently even in the case of fresh material, it is necessary to determine also the ammonia and nitrate nitrogen; this is done by extraction of an aliquot portion of the material with a normal solution of NaCl or KCl, adding NaOH or MgO, and distilling or aerating to obtain the ammonia; the nitrate can then be determined in the residual solution, by the reduction method. For the complete analysis, two or three portions of the dry material are employed. These are treated first with ether, to give the ether soluble fraction; with cold water to give the water soluble organic matter, sugar, ash and nitrogen; with hot water, for determining the soluble organic matter, ash and nitrogen; with hot alcohol, to give the alcohol-soluble fraction; with dilute (2 per cent) hydrochloric acid, at boiling temperature for 5 hours, to give the hemicelluloses, determined as reducing sugars in the extract; known portions of the residue are then treated with ten volumes of 80 per cent sulfuric acid in the cold for 2 to 3 hours, followed by diluting with 15 volumes of water and boiling for 5 hours, to give the cellulose, determined as reducing sugar in the extract; the final residue, after the last treatment, is analyzed for ash and nitrogen, to enable one to calculate the lignin content.<sup>1</sup> Frequently, the hemicelluloses or pentosans only, the cellulose and the lignin are determined on separate samples of material.<sup>2</sup>

Table 66 (see also table 30, p. 372) gives a series of typical analyses of some common plant materials which usually reach the soil. Some of the materials, like the pine needles (*Pinus strobus*), are very high in fats, waxes and oils, as shown by the ether and alcohol soluble fractions; others are high in proteins, as in the case of the two leguminous plants; still others, as the cereal straw and the corn stalks, are high in cellulose and hemicelluloses; some of the materials are high in lignin, as in the case of the oak leaves, pine needles and straw. All of these constituents markedly influence the rapidity and nature of the decomposition processes that the plant residues undergo in soil or in compost and the products of decomposition which result.

<sup>1</sup> Waksman, S. A. and Tenney, F. G. Soil Sci., **24**: 275-283. 1927; **26**: 155-171. 1928; **28**: 55-84. 1929; Jour. Ind. Engin. Chem. Anal. Ed., **2**: 167-173. 1930.

<sup>2</sup> Dore, W. H. Jour. Ind. Engin. Chem., **12**: 476-480, 984. 1920; Schorger, A. W. Ibid., **9**: 556-560, 561-565. 1917; Schwalbe, C. J. Die chemische Untersuchung pflanzlicher Rohstoffe und der daraus abgeschiedenen Zellstoffe. Berlin. 1920. ..



*Influence of age of plant upon its composition.* Among the various factors that influence the rapidity of decomposition of plant materials, the age of the plant occupies a prominent place. Although the plant continues to assimilate nutrients from the soil until it reaches maturity,

TABLE 65  
*Proximate chemical composition of various plant materials*  
On per cent basis of dry material

CHEMICAL CONSTITUENT	WHEAT STRAW	SOY- BEAN MEAL	MATURE OAK LEAVES	DEAD PINE NEEDLES	CORN STALKS	ALFALFA TOPS
Ether-soluble fraction.....	1.10	3.80	3.85	11.37	1.75	2.75
Cold water soluble organic matter..	5.57	22.09	8.28	4.42	10.58	12.44
Hot water soluble organic matter..			5.73	2.86	3.56	4.80
Alcohol soluble fraction.....			5.92	12.58	4.19	7.66
Hemicelluloses.....	26.35	11.08	17.97	17.10	21.91	13.14
Cellulose.....	39.10	28.53	12.78	14.79	28.67	23.65
Lignin.....	21.60	13.84	24.76	21.89	9.46	8.95
Crude protein.....	2.10	11.04	4.25	2.12	2.44	12.81
Ash.....	3.53	9.14	5.10	2.51	7.54	10.30

TABLE 67  
*Influence of age of rye plant on its chemical composition*  
On per cent basis of dry material

PLANT CONSTITUENTS	PLANTS 10-14 INCHES HIGH, FIRST CUTTING	HEADS JUST BEGIN TO FORM, SECOND CUTTING	JUST BEFORE BLOOM,* THIRD CUTTING	MATURE PLANTS,* FOURTH CUTTING
Fats and waxes.....	2.60	2.60	1.70	1.26
Soluble in cold water.....	34.24	22.74	18.16	9.90
(Reducing sugars).....	(3.46)	(5.98)	(2.75)	(2.05)
Pentosans.....	16.60	21.18	22.71	22.90
Cellulose.....	18.06	26.95	30.59	36.29
Lignin.....	9.90	11.80	18.00	19.80
Total nitrogen.....	2.50	1.76	1.01	0.24
(Water soluble nitrogen)....	(0.74)	(0.48)	(0.28)	(0.07)
Ash.....	7.66	5.90	4.90	3.90

\* Stems and leaves only.

the percentage of nitrogen and the mineral content of the plant reach a maximum at an early stage of growth; they then begin to diminish, reaching a minimum relative concentration at maturity. In addition to the total and water-soluble nitrogenous compounds, the young plant

is also rich in soluble sugars.<sup>3</sup> In the case of various grasses, there is, with an increase in maturity, a decrease in the content of water-soluble substances, minerals, fats, proteins and an increase in the cellulose, hemicelluloses and lignin,<sup>4</sup> as shown in table 67. This is true not only of the Gramineae but also of the Leguminosae<sup>5</sup> and even of leaves and needles of trees.<sup>6</sup> It is not the actual age of the plant, however, which influences its composition but the degree of its relative maturity.<sup>7</sup>

These results have a very important bearing upon the rapidity of decomposition of the plant residues in the soil and upon their use for green manuring purposes, as shown in fig. 47. The younger the plant, the more rapidly it decomposes in the soil.<sup>8</sup> This was explained by the diminishing nitrogen content of plants with the increase in maturity;<sup>9</sup> since legumes contain more nitrogen than non-legumes, they decompose more rapidly. The rapidity of decomposition is accompanied by a more rapid formation of nitrates (a in fig. 47);<sup>10</sup> it has been suggested that the water-soluble nitrogen, rather than the total nitrogen of the plant, is largely responsible for this phenomenon.<sup>11</sup> Drying of green plants was found<sup>12</sup> to retard the rapidity of their decomposition; this was believed

<sup>3</sup> Bogdanov, S. Selsk. Khoz. i. Liesovod., 193: 227-271. 1899; Singleton, G. H. N. J. Agr. Exp. Sta. Bul., 421. 1925.

<sup>4</sup> Hunt, T. F. Ill. Agr. Exp. Sta. Bul., 5. 1899; Berry, R. A. Jour. Agr. Res., 10: 360. 1920; Wilfarth, H., Romer, H. and Wimmer, G. Landw. Vers. Sta., 63: 1-70. 1906; Beckmann, E., Liesche, O. and Lehmann, F. Biochem. Ztschr., 139: 491-508. 1923; Waksman, S. A. and Tenney, F. G. Soil Sci., 24: 317-333. 1927; Berry, R. A. Jour. Agr. Sci., 10: 358. 1920.

<sup>5</sup> König, J., Fürstenberg, A. and Murdfeld, R. Landw. Vers. Sta., 65: 55-110. 1907; Leukel, W. A., Barnette, R. M. and Hester, J. B. Soil Sci., 28: 347-371. 1929.

<sup>6</sup> Fricke, K. Ztschr. physiol. Chem., 143: 272-289. 1925; Combes, R. Compt. Rend. Acad. Sci., 184: 533-535. 1927; Piney, M. M. Rev. Gen. Bot., 41: 67-94. 1929; Gäumann, E. Flora, 123: 344-385. 1928.

<sup>7</sup> Bal, D. V. Agr. Jour. India, 17: 133-155. 1922; see also Erdman, L. W. Jour. Amer. Soc. Agron., 21: 361-366. 1929.

<sup>8</sup> Maynard, L. N. Y. (Cornell) Agr. Exp. Sta. Bul., 394. 1917; Martin, T. L. Ibid., 406. 1921; Merkle, F. G. Jour. Amer. Soc. Agron., 10: 281-302. 1918; Hutchinson, C. M. and Milligen, S. Agr. Res. Inst. Pusa. Bul., 40. 1914. The practical phases of green manuring are discussed by A. J. Pieters. Green manuring, principles and practice. J. Wiley, New York. 1927.

<sup>9</sup> Hill, H. H. Jour. Agr. Res., 33: 77-99. 1926.

<sup>10</sup> Leukel, Barnette and Hester. Soil Sci., 28: 347-371. 1929.

<sup>11</sup> Whiting, A. L. Jour. Amer. Soc. Agron., 18: 854-876. 1926; Soil Sci., 24: 31-39. 1927.

<sup>12</sup> Whiting, A. L. and Schoonover, W. R. Soil Sci., 9: 137-149. 1920.

to be due either to a change in the physical condition of the plant, to a loss of water absorptive capacity, or to a change of the soluble hemicelluloses into less soluble forms.

*Decomposition of organic matter in soil by microorganisms.* When organic matter in the form of complex plant or animal residues is added to the soil, it is acted upon by various groups of microorganisms, including fungi, actinomyces and bacteria. There is no doubt that some of the protozoa as well as the worms and insects living in the soil ingest the undecomposed or partially decomposed organic matter, utilizing the

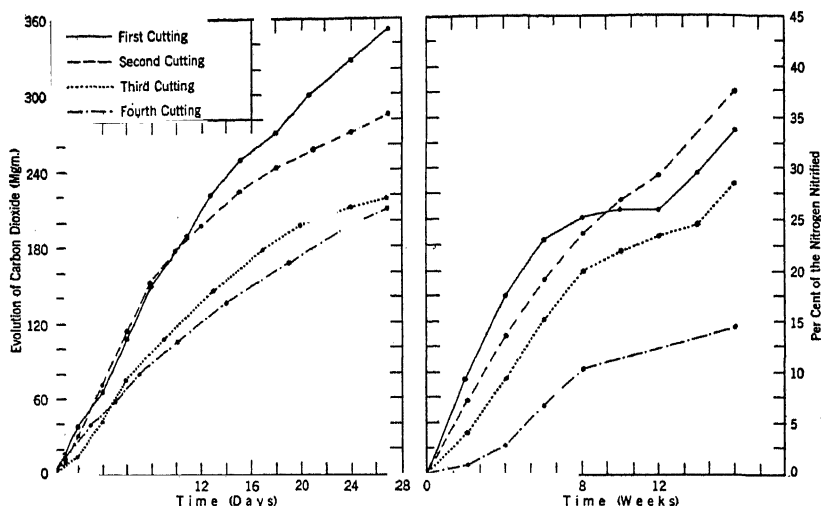


FIG. 47. Decomposition of plants harvested at different stages of growth, as measured by the liberation of CO<sub>2</sub> from rye plants (left—from Waksman and Tenney) and formation of nitrate from *Crotalaria* (right—from Leukel, Barnette and Hester).

various chemical constituents as nutrients; a number of physical and chemical changes are thereby brought about, the extent of which depends upon the nature of the organism and the environmental conditions. As a result of these activities, a number of the constituents of the organic matter are broken down by processes of hydrolysis, oxidation, reduction, and condensation. These processes make available the locked up energy, which is utilized by the microorganisms for their activities. The organic matter is thereby mineralized and the inorganic compounds of nitrogen, phosphorus, potassium, calcium, magnesium, etc. are liberated in forms available for plant growth. These inorganic

substances can be looked upon more as waste products of microbial metabolism, since they are either present in the plant and animal residues in excess of what is required by the microorganisms for the building up of their own cell substance, or they are liberated as a result of the decomposition of the microbial cells.

A part of the organic matter, consisting of the monosaccharides, some of the pentosans and hexosans, the proteins and their derivatives, is completely decomposed, with the formation of  $\text{CO}_2$ ,  $\text{H}_2\text{O}$ ,  $\text{NH}_3$  and various minerals, under aerobic conditions;  $\text{CH}_4$  and  $\text{H}_2$  are also produced under anaerobic conditions. A part of the material decomposed is reassimilated by the organisms and synthesized into microbial protoplasm: under aerobic conditions this amounts to as much as 20 to 40 per cent of the decomposed material. A part of the organic complexes undergoing decomposition is left in the form of intermediate products, due either to the greater resistance of these to the action of the specific microorganisms, or to the formation, under certain conditions such as excessive moisture and acidity, of products which hinder further development of the organisms. Some of the constituents of the original organic matter, consisting largely of fats, waxes, tannins, resins, certain hexosans, and lignin, are left undecomposed. This mass of undecomposed, partially decomposed and transformed materials makes up the soil organic matter, which is being modified constantly. A large part of this organic matter is soluble in alkalis and is commonly referred to as "humus" or the "humified" fraction of the organic matter.

The chemical ingredients of the organic matter added to the soil are decomposed at various rates and to varying degrees. Of the non-nitrogenous substances, the monosaccharides are the first to disappear; these are followed by the starches and pectins and then by the cellulose and pentosans. The lignin, waxes and tannins are decomposed only very slowly. The more ripe and mature the plant, the greater is the degree of its lignification and the more slowly does the lignified portion decompose. The pentosans disappear at first somewhat more rapidly than the cellulose. The differences in the rapidity of decomposition of various organic substances (fig. 48) can be readily explained by the differences in their chemical composition.

Following the decomposition of plant residues with a low nitrogen content, there is an increase in the relative concentration of organic nitrogen and carbon in the residual material, due to the fact that the pentosans and cellulose which are of a low carbon content (42 to 44 per cent) disappear, while the lignin as well as the synthesized cell substance

with a high carbon content persist.<sup>13</sup> The proteins introduced into the soil with the organic matter are first hydrolyzed to amino acids; some of the amino acids are more rapidly and completely decomposed than others. As a result of incomplete oxidation, substances may be formed

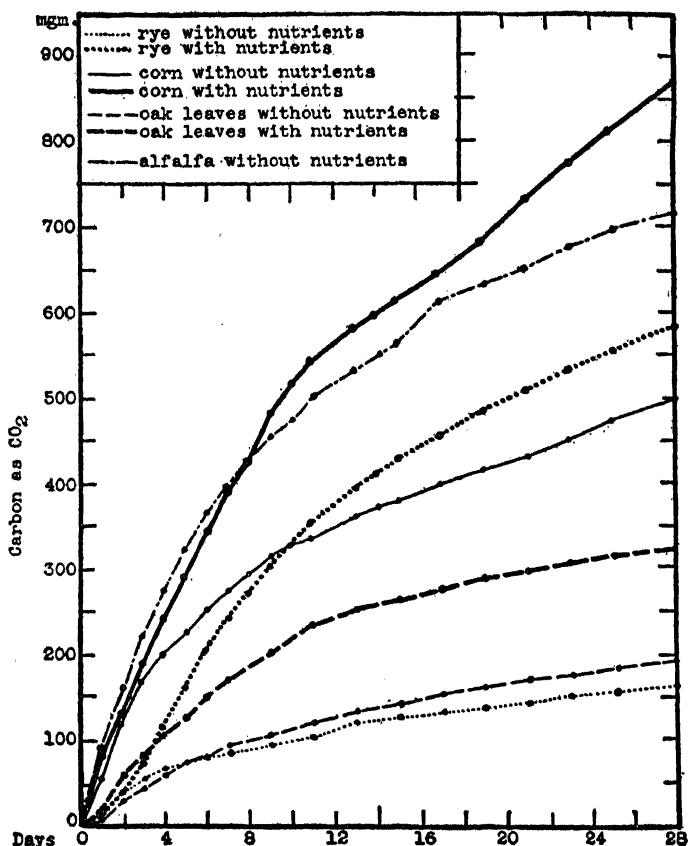


FIG. 48. Course of decomposition of rye straw, corn stalks, alfalfa tops, and oak leaves, with and without additional inorganic nutrients, as shown by the evolution of CO<sub>2</sub> (from Waksman and Tenney).

which are more resistant than the original materials. In view of the fact, however, that constant synthesis of microbial protoplasm takes place, it is often difficult to establish how much of the protein has been

<sup>13</sup> König, J. *Biochem. Ztschr.*, 171: 261-276. 1926.

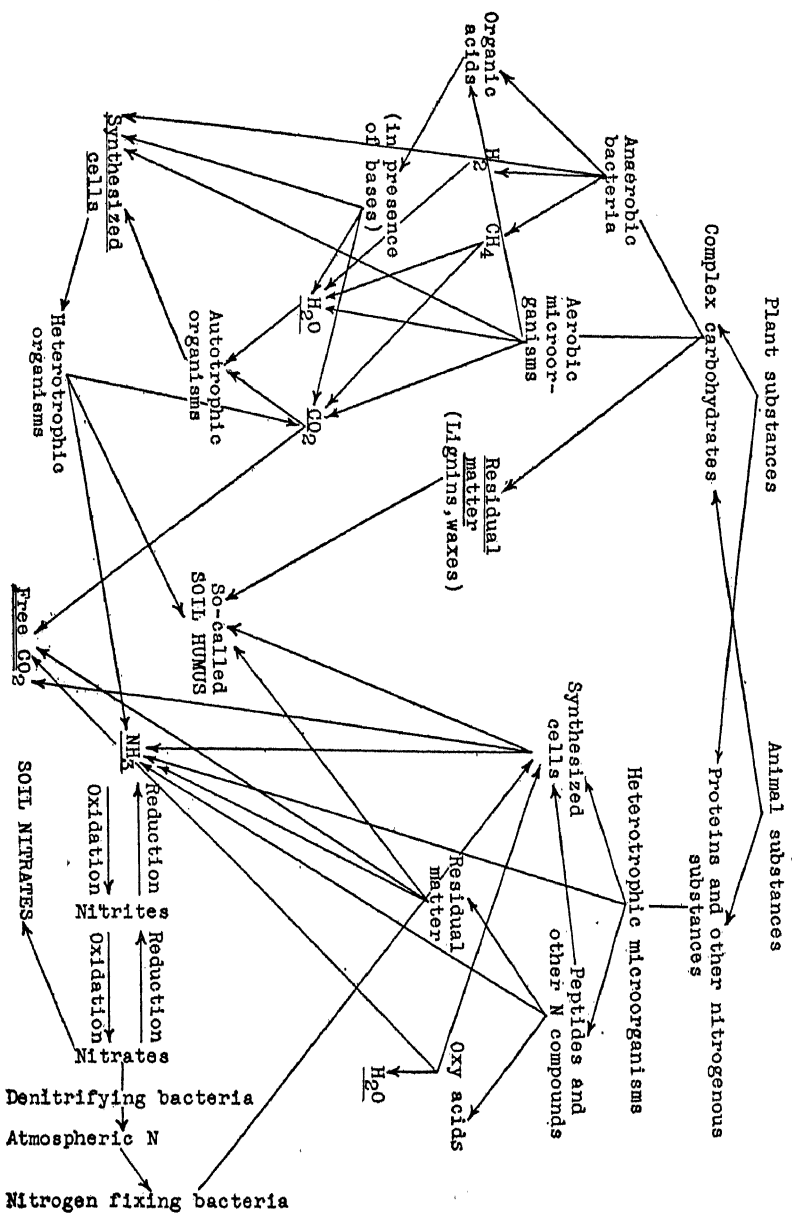


Fig. 49. Decomposition of organic matter in the soil by microorganisms (original).

decomposed and how much has been assimilated by the microorganisms. Under aerobic conditions, especially in aerated and acid soils, the fungi are active. Fungi attack the organic matter rapidly, especially in the presence of sufficient available nitrogen, and synthesize large quantities of mycelium; this is of course later decomposed by the bacteria, with the result that the nitrogen is liberated again as ammonia and rapidly changed to nitrate. The bacteria and actinomyces, which develop in the less acid aerated soils, also attack the proteins readily, liberating ammonia even before all the carbohydrates have been decomposed, a process uncommon for most fungi. Bacteria leave a larger amount of the material in the form of intermediate products, including the various organic acids.

The extent of decomposition of carbohydrates by microorganisms is found to depend not only on the nature of the organism, but also on the nature and amount of available nitrogen. For every unit of carbon decomposed as a source of energy, a certain amount of nitrogen is assimilated, whether the latter is present in the form of proteins, simple protein degradation products or inorganic salts. Fungi assimilate a larger amount of the carbon decomposed and, although their nitrogen requirement is less than that of bacteria, the total quantity of nitrogen assimilated is much greater, because of the comparatively much smaller amount of protoplasm synthesized by the bacteria. Under anaerobic conditions, as in water-logged soils, various organic acids will be formed from the carbohydrate. When green manures are used in tropical countries, these conditions will favor a greater preservation of the nitrogen and of organic matter in general.<sup>14</sup> A schematic presentation of the transformation of a typical protein in the soil is given in fig. 50.<sup>15</sup>

The decomposition of organic matter in soil can be followed either by determining one or more of the products of the reaction, at a definite set of environmental conditions, and with a definite knowledge of the nature of the organisms which are active, or by measuring the disappearance of the various chemical complexes present in the original organic material. These methods can be classified into three groups. (1) Methods which are based upon measuring the amount of total original organic matter decomposed or only of one or more of its constituents, such as cellulose or pentosan. (2) Those methods which are based upon measuring the accumulation of one of the final products of

<sup>14</sup> Subrahmanyam, V. *Jour. Agr. Sci.*, 19: 627-648, 649-655. 1929; Joachim, A. W. R. and Kandiah, S. *Trop. Agr. Ceylon*, 72: 253-271. 1929.

<sup>15</sup> Schreiner, O. *Jour. Amer. Soc. Agron.*, 18: 115-126. 1926.

the reaction: viz., carbon dioxide, ammonia or nitrate. These are the easiest and most reliable methods; carbon dioxide is the end product of energy utilization and respiration of microorganisms;  $\text{NH}_3$  is the product of the nitrogen metabolism of heterotrophic microorganisms; since some or all of the ammonia is rapidly oxidized in the soil to nitrates,

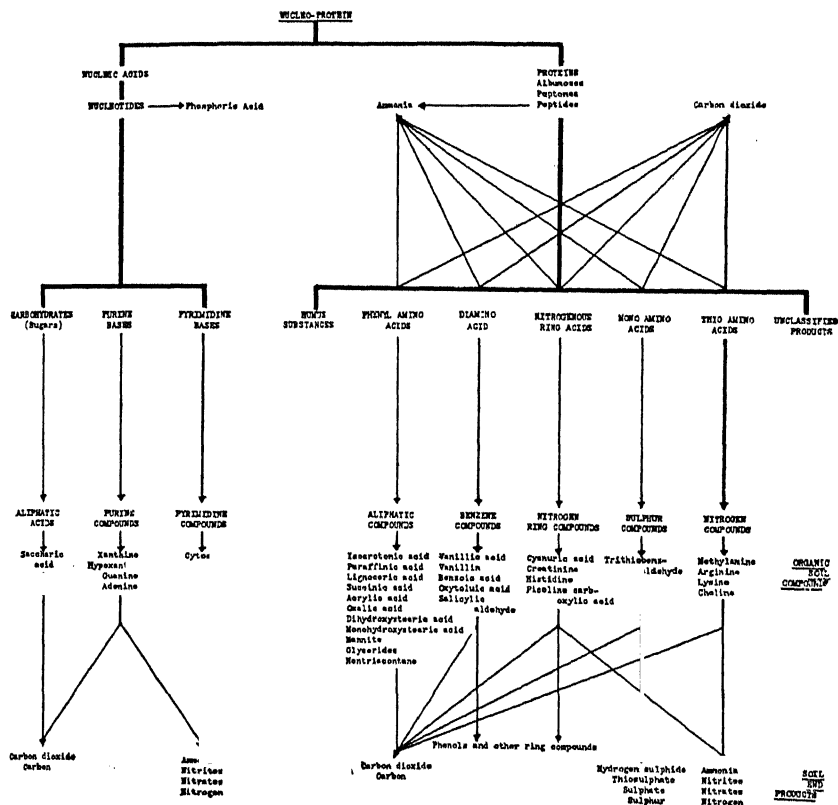


FIG. 50. Decomposition of a complex protein in the soil by microorganisms (from Schreiner).

both ammonia and nitrates should be determined. (3) Methods for measuring the formation or accumulation of the less readily decomposable organic materials in the soil, the soil humus.

*Decomposition of organic matter, as measured by the disappearance of its various chemical constituents.* Microorganisms do not decompose an organic substance as a whole, but attack only certain definite consti-



uents, some in preference to others. Therefore, the disappearance of a certain part of plant or animal material as a result of its decomposition by a pure culture of an organism or mixed cultures tells very little concerning the nature of the transformation that has taken place. It is essential to determine just what chemical constituents have been de-

TABLE 68

*Decomposition of mature rye straw, as indicated by its composition at the beginning and at end of decomposition*

	AT BEGINNING OF EXPERIMENT	MATERIAL LEFT AT END OF EXPERIMENT	
	mgm.	mgm.	per cent of original
Organic matter (free from water-soluble substances and ash).....	15,114	8,770	58.03
Pentosan.....	3,928	1,553	39.54
Cellulose.....	6,262	2,766	44.17
Lignin.....	3,403	3,019	88.72
Protein.....	181	519	286.70

TABLE 69

*Decomposition of plant materials in the presence of additional inorganic nitrogen compounds and phosphates*

Per cent of total material left

PLANT CONSTITUENT	ORGANIC MATTER					
	Rye straw			Corn stalks		
	At start	At end of experiment		At start	At end of experiment	
		No nutri- ents	Nutri- ents added		No nutri- ents	Nutri- ents added
Total dry organic matter.....	100	93.1	71.4	100	76.5	57.1
Hemicelluloses.....	25.9	23.6	12.8	23.7	15.2	7.8
Cellulose.....	35.4	34.4	20.1	31.0	22.1	11.9
Lignin.....	13.0	12.1	11.8	10.2	10.1	9.1

composed by the given organism and under a given set of conditions. This is clearly brought out in table 68, in which the results on the decomposition of mature rye straw in the presence of additional inorganic nitrogen for a period of 2 months are reported. The pentosan has disappeared more rapidly than the cellulose; both groups of carbohydrates were decomposed at a greater rate than the total organic matter.

This is due to the fact that the lignin has decomposed only to a very limited extent while the proteins have increased considerably.

The effect of additional inorganic nitrogen and phosphate upon the

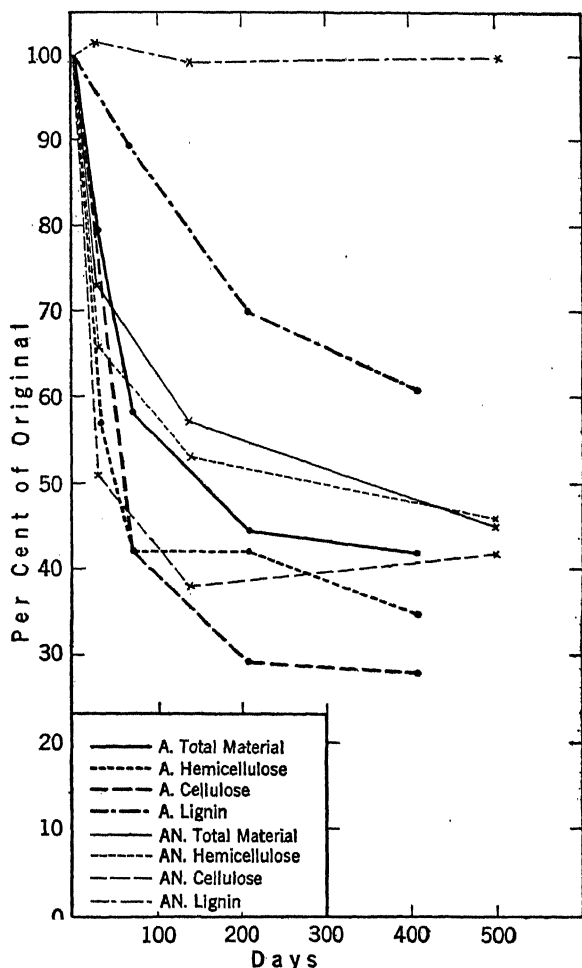


FIG. 51. Course of decomposition of alfalfa plant and its chemical constituents, under aerobic (A) and anaerobic (AN) conditions (from Tenney and Waksman).

decomposition of the two most important groups of carbohydrates, namely the hemicelluloses and cellulose is shown in table 69; the plant residues were decomposed under aerobic conditions for a period of 4

weeks at 27°C. Nearly twice as much of each group of carbohydrates was decomposed in the presence of additional inorganic salts. The increased decomposition of the total organic matter, due to available nitrogen, could be accounted for in both cases by the decomposition of the cellulose and hemicelluloses. The lignin was not affected to any extent, this group of plant constituents being most resistant to decomposition as shown elsewhere.

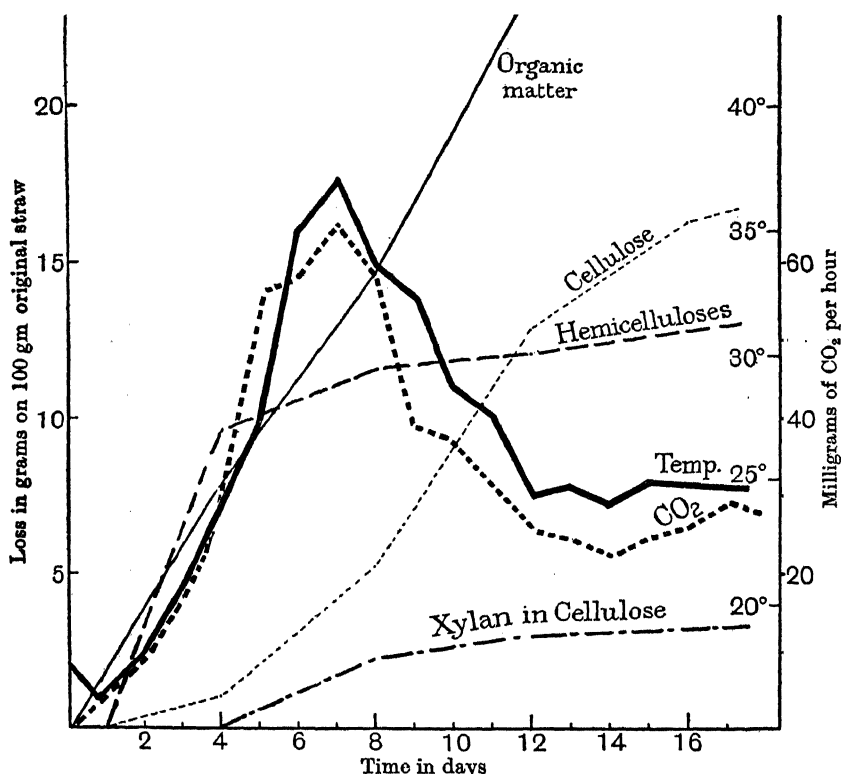


FIG. 52. Decomposition of oat straw, as shown by the evolution of  $\text{CO}_2$ , temperature change and loss of chemical constituents (from Norman).

A summary of the processes taking place in the decomposition of the various chemical constituents of alfalfa under aerobic and anaerobic conditions is shown in fig. 51.<sup>16</sup> The total organic matter was reduced,

<sup>16</sup> Tenney, F. G. and Waksman, S. A. *Soil Sci.*, **28**: 55-84. 1929; **30**: 143-160. 1930.

under aerobic conditions, to 38.5 per cent of the original in 405 days. The cellulose has been reduced to 23 per cent, the hemicelluloses to 37 per cent, while the lignin has decreased to the least extent, namely 57.4 per cent. Under anaerobic conditions, the total material was reduced, in 498 days, to 45.4 per cent; the cellulose has decreased to 42.7 per cent, the hemicelluloses to 46.0 per cent, while the lignin was preserved practically quantitatively.

Fig. 52<sup>17</sup> shows the rate of decomposition of oat straw and its constituents, as compared with the course of CO<sub>2</sub> evolution and temperature

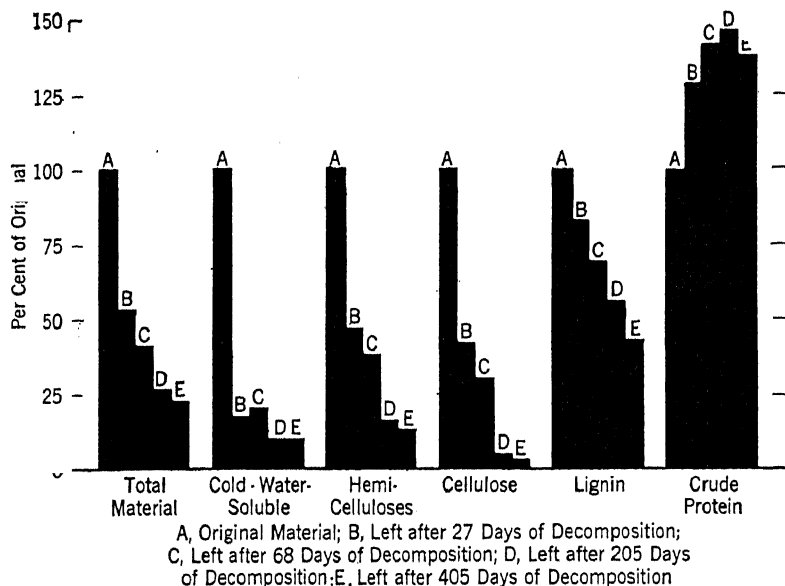


FIG. 53. Course of decomposition of various chemical constituents of corn stover under aerobic conditions (from Tenney and Waksman).

change. The hemicelluloses decomposed faster than the cellulose, at the beginning, but after a few days, the rate of the decomposition of the cellulose superseded that of the hemicelluloses. Fig. 53<sup>16</sup> shows the relative decomposition, under aerobic conditions, of the different plant constituents of corn stover. At the end of 405 days, the cellulose was practically all decomposed, while the lignin has resisted decomposition to the greatest extent, and the protein actually increased in total amount, at the expense of the inorganic nitrogen added to the compost.

<sup>17</sup> Norman, A. G. *Ann. Appl. Biol.*, 17: 575-613. 1930.

*Origin and nature of humus or soil organic matter.* As a result of the decomposition of plant and animal residues added to the soil, there is produced in course of time, a dark-colored material, usually referred to as humus and possessing certain characteristic properties. It is low in cellulose, fairly high in hemicelluloses (rich in uronic acids), contains some fatty, waxy and resinous substances and is especially high in organic nitrogenous compounds, or protein-like complexes, and in modified lignin complexes, as shown in Table 70.<sup>18</sup> A large part of this humus is soluble in alkalis, and precipitated on neutralization of the solution with an acid. The various preparations obtained by the alkali solution and the acid precipitation have been given different names,

TABLE 70

*Chemical composition of the organic matter in different mineral soils*

On per cent basis of total soil organic matter (C  $\times$  1.72)

SOIL NUMBER	pH OF SOIL	TOTAL ORGANIC MATTER BY IGNITION	TOTAL CARBON $\times$ 1.72	TOTAL NITROGEN	C/N RATIO	ETHER-SOLUBLE	ALCOHOL SOLUBLE	HEMICELLULOSES	CELLULOSE	LIGNIN-HUMUS COMPLEX*	PROTEIN
4	6.8	7.89	4.49	0.242	10.8	3.56	0.58	5.44	3.55	43.37	33.78
6	7.6	5.98	2.74	0.154	10.3	4.71	1.53	8.60	5.22	40.81	34.74
16	6.4	17.10	11.20	0.670	9.9	0.80	0.82	5.53	4.12	41.87	37.35
18	8.3	10.34	6.24	0.332	10.9	1.02	0.88	6.96	3.50	42.05	33.25
21	8.3	10.06	7.40	0.395	10.9	0.46	0.84	8.54	2.83	42.83	33.36
29	7.8	10.16	6.48	0.315	12.0	0.62	0.61	8.21	3.64	49.29	30.38

\*Amount of  $\frac{\text{C in H}_2\text{SO}_4 \text{ residue} \times 100}{\text{total carbon}} - \frac{\text{protein (N} \times 6.25) \text{ in residue} \times 100}{\text{total organic matter}}$

ranging from "humic acid," "ulmic acid," "humins," and "ulmins" to "crenic acid" and "apocrenic acid." These names stand, however, not for definite chemical compounds, but for preparations, which vary qualitatively and quantitatively with the soil from which they are obtained, method of preparation, reagents used, etc.<sup>19</sup>

The term "humus" will be applied to the soil organic matter as a whole. It is best calculated by multiplying the organic carbon content

<sup>18</sup> Waksman, S. A. Proc. 2d Comm. Intern. Soc. Soil Sci. Budapest. 1929, Part A; Soil Sci., 30: 97-116. 1930.

<sup>19</sup> Oden, S. Kolloidchem. Beih., 11: 75-260. 1919; Waksman, S. A. Soil Sci., 22: 123-162, 221-232. 1926; Cellulosechem., 11: H 10/11. 1930.

of the soil by 1.724. A number of definite chemical compounds can be readily isolated from this humus, as shown by Schreiner and Shorey.<sup>20</sup> These compounds are either constituents of the plant material undergoing decomposition, or are modified complexes, or constituents of the synthesized microbial cell substance. They include various carbohydrates, aldehydes, organic acids, alcohols, fats, waxes, esters, various nitrogenous compounds (pyridine and pyrimidine derivatives, purine bases, amines, amino acids), pectins and lignin derivatives. This humus is much more resistant to decomposition than the original plant and animal substances from which it originated.

Another characteristic property of the soil humus is the definite relation between its carbon and nitrogen content. This ratio usually ranges from 8:1 to 12:1, in the case of inorganic soils, becoming narrower

TABLE 71

*Influence of carbon-nitrogen ratio in soil upon the decomposition of soil organic matter (from Sievers and Holtz)*

CARBON	NITROGEN	C:N RATIO	CARBON LIBERATED AS CO <sub>2</sub>	NITROGEN LIBERATED AS NITRATE	C:N RATIO OF MINERALIZED ELEMENTS
<i>per cent</i>	<i>per cent</i>		<i>mgm.</i>	<i>p.p.m.</i>	
0.910	0.091	10.0	119.6	15.4	7.77
1.684	0.143	11.8	187.8	18.3	10.26
1.860	0.155	12.0	167.5	17.6	9.52
2.889	0.233	12.4	230.9	26.3	8.78

with depth of soil.<sup>21</sup> The wider the C:N ratio, the more readily will the humus decompose, under normal conditions, as shown in Table 71.

The nature and abundance of humus in soil will be influenced by climatic conditions, especially temperature, precipitation and evaporation, which determine the nature and abundance of the surface vegetation and the decomposition processes. The lower the temperature and the higher the humidity, the greater will be the excess of accumulation of plant residues over their decomposition and the wider will be the C:N

<sup>20</sup> Schreiner, O. and Shorey, E. C. *Bur. Soils, U. S. Dept. Agr. Bul.*, **53**, **74**. 1909-1910; Shorey, E. C. *Ibid.*, **Bul.**, **88**. 1913; Lathrop, E. C. *Soil Sci.*, **1**: 509-532. 1916; Morrow, C. A. *Thesis, Univ. Minn.* 1918.

<sup>21</sup> Berthelot (cited by Dehérain. 1902, p. 767); Sievers, F. J. and Holtz, H. F. *Wash. Agr. Exp. Sta. Bul.*, **166**. 1922; **176**. 1923; **206**. 1926; Brown, P. E. and O'Neil, A. M. *Iowa Agr. Exp. Sta. Bul.*, **75**. 1923.

ratio of the humus resulting.<sup>22</sup> Fig. 54 shows such relation for a number of different soils.

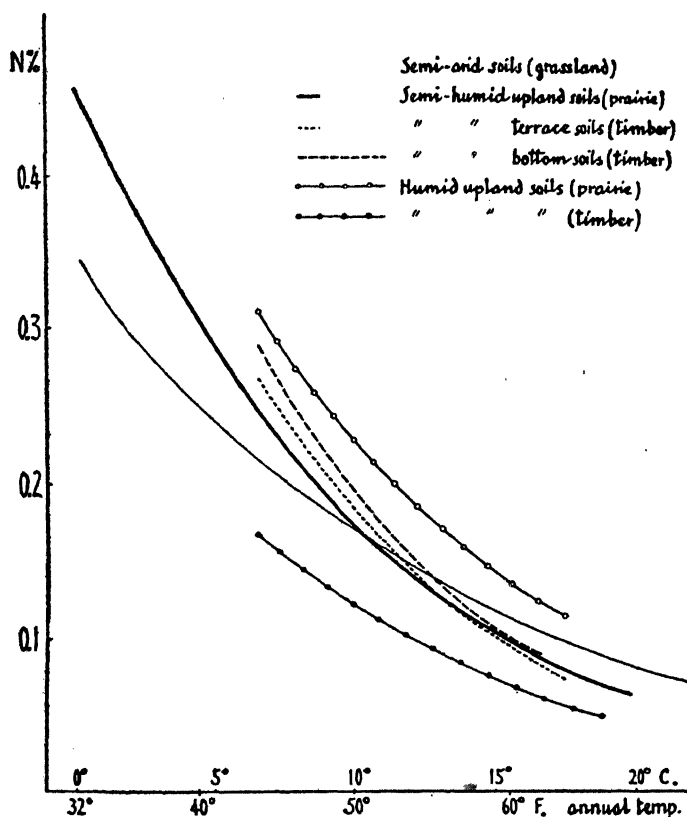


FIG. 54. Influence of mean annual temperature of region upon the nitrogen content of the soil (from Jenny).

*Soil humus and the activities of microorganisms.* Organic matter influences the growth and activities of microorganisms in the soil by forming a more favorable physical and chemical environment and by offering a source of energy and other nutrients. The different constituents of the organic matter and the degree of their decomposition influence to

<sup>22</sup> Mohr, E. C. J. The soils of Java and Sumatra. Amsterdam, deBussy. 1922; Sensiust, M. W. Amer. Soil Survey Assn. Rpt. Bul., 6: 149-161. 1925; Jenny, H. Jour. Amer. Soc. Agron., 20: 900-912; Soil Sci., 27: 169-188. 1928; Missouri Agr. Exp. Sta., Res. Bul., 152. 1930. Waksman, S. A. and Gerretsen, F. C. Ecology, 12: 33-66. 1931.

a large extent the nature of the organisms which are capable of developing. The favorable influence of small amounts of soil extract upon the activities of various bacteria, like *Azotobacter*, led to various assumptions that we are dealing with certain vitamins (auximones).<sup>23</sup> It was also suggested that the favorable influence of organic matter upon the growth of various microorganisms is due to the chemical improvement of the medium. Others considered soil humus as a reservoir of nutrients required by the soil organisms.<sup>24</sup>

Hoppe-Seyler<sup>25</sup> believed that "humic" substances afford a habitat and substrate for the soil bacteria, fungi, algae and lower animals, but that "humus" itself cannot offer any food to plants, or animals and cannot be decomposed by bacteria. The very fact that humus may accumulate in the soil indicates the low availability of these compounds as sources of energy for microorganisms. One could hardly expect that sources of available energy should exist in the soil in great abundance in the presence of the various microorganisms and not be attacked. The low availability of the soil organic matter as a source of carbon is further confirmed by the fact that the addition of a small amount of available nitrogen, such as  $\text{NaNO}_3$  or  $(\text{NH}_4)_2\text{SO}_4$ , does not greatly stimulate decomposition of the organic matter, as indicated by the evolution of carbon dioxide, except in soils with a wide carbon-nitrogen ratio. The nitrogenous part of the soil organic matter seems to be more available for the activities of microorganisms than the carbon part. On adding available energy, even in the form of celluloses, various organisms are enabled to use some of the soil nitrogen. This has also been clearly demonstrated by various investigators,<sup>26</sup> who cultivated fungi using purified "humus" as the only source of nitrogen; the "humus" could not be utilized, however, as a source of carbon. The various claims put forth that "humus" can be used as a source of energy for nitrogen fixing bacteria, as well as for fungi and urea bacteria still need confirmation.<sup>27</sup>

<sup>23</sup> See p. 370; also Burk, D. and Lineweaver, H. *Jour. Bact.*, 19: 389-414. 1930.

<sup>24</sup> Kaserer, H. *Inter. Mitt. Bodenk.*, 1: 367-375. 1911.

<sup>25</sup> Hoppe-Seyler, F. *Ztschr. physiol. Chem.*, 13: 66-121. 1889.

<sup>26</sup> Reinitzer, F. *Bot. Ztg.*, 58: 59-73. 1900; Nikitinsky, J. *Jahrb. wiss. Bot.*, 37: 365-420. 1902.

<sup>27</sup> It is sufficient to cite the work of Robertson, R. A., Irvine, J. C. and Dobson, M. E. *Biochem. Jour.*, 2: 458-480. 1907; Christensen, H. *Centrbl. Bakt. II*, 24: 130. 1909; Krzemieniewski, 1908 (p. 508); Pringsheim, 1908-1912 (p. 495). The same is true of the results of Lipman and Teakle, on the use of displaced soil solution and residual soil as sources of energy by *Azotobacter*. *Soil Sci.*, 19: 99-103. 1925.



Warmbold<sup>28</sup> demonstrated previously that humus cannot serve as a source of energy for nitrogen-fixing bacteria. Even crude "humic acids" (extracted with NaOH and precipitated with HCl) cannot be used as nutrients by the majority of microorganisms, but may stimulate the activities of various organisms in a physico-chemical way as accompanying mineral impurities will do.<sup>29</sup> It is true, however, that humus does decompose in well aerated and limed soils and that it does not accumulate under these conditions.

When the soil is heated, treated with antiseptics, or air dried, its organic matter decomposes more readily than that in the untreated soil. The amount of CO<sub>2</sub> formed from sterilized soil inoculated with a suspension of bacteria or fungi may run parallel to the availability of the organic matter in the soil. König and Hasenbäumer<sup>30</sup> determined the amount of CO<sub>2</sub> evolved from 500 grams of soil in seven weeks, after the soil was sterilized and inoculated with pure cultures of some microorganisms.

	CO <sub>2</sub> mgm.
Sterile soil.....	60.7
<i>Bac. ramosus</i> .....	179.2
<i>Bac. vulgatus</i> .....	273.8
<i>Act. chromogenus</i> .....	156.0

The evolution of CO<sub>2</sub> by a pure culture of an organism from the soil itself, to which no fresh organic matter has been added, varies not only with the organism but with the amount and availability of the organic matter in the soil. *Trichoderma* was found<sup>31</sup> to produce in eight days, from 100 grams of sterile sandy loam manured with cow manure each year, 124.1 mgm. CO<sub>2</sub>, while only 37.4 mgm. were produced from the same quantity of the corresponding unfertilized soil. These quantities are much larger than those obtained by König and Hasenbäumer because of the difference in composition of soil used and the greater activity of the particular fungus as compared with the bacteria. The fact that a soil kept at optimum moisture and temperature conditions gives off a constant stream of CO<sub>2</sub> indicates that the soil organic matter is constantly decomposing, truly at a low rate, but very definitely. Among the various soil organisms, certain actinomyces and various non-

<sup>28</sup> Warmbold, H. Landw. Jahrb., 35: 1-123. 1906.

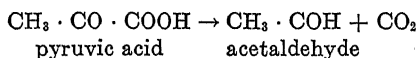
<sup>29</sup> Ritter, G. A. Intern. Mitt. Boden., 2: 301-311. 1912. Centrbl. Bakt. II, 34: 577-666. 1912.

<sup>30</sup> König and Hasenbäumer, 1920 (p. 544).

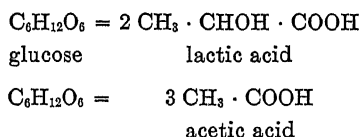
<sup>31</sup> Waksman and Starkey, 1924 (p. 702).

spore forming bacteria and cocci seem to be especially capable of attacking the soil humus.

*Evolution of carbon dioxide as an index of decomposition of organic matter in the soil.* In determining the ability of the soil microorganisms to decompose organic matter by the rapidity of evolution of  $\text{CO}_2$ , the terms "oxidative capacity," "carbon dioxide producing capacity," "respiratory capacity of soils" are often used. They all designate the decomposition of organic matter in the soil by microorganisms, whereby energy is liberated. However, carbon dioxide may be formed without the liberation of energy, as in the decomposition of pyruvic acid:



On the other hand, energy may be liberated without the formation of carbon dioxide, as in the case of the anaerobic fermentations of sugars:



Some  $\text{CO}_2$  undoubtedly also originates in normal soils from carbonates interacting with organic or mineral acids formed by biological agencies.

Kissling and Fleischer,<sup>32</sup> as far back as 1891, used  $\text{CO}_2$  production of peat soils as an index of the rapidity of decomposition going on in the soil. The addition of sand was found to stimulate oxidation greatly, while temperature was found to be one of the most important factors. As a measure of oxidation taking place in the soil, Dehérain and Demoussy<sup>33</sup> used the amount of  $\text{CO}_2$  present in the atmosphere of a closed 100-cc. tube containing the soil. The tube of soil was kept at constant temperature for a certain period of time. They demonstrated that the production of carbon dioxide in basic soil (1) is due almost wholly to bacteria; (2) it increases with temperature to about  $65^\circ\text{C}$ ., then decreases, and at higher temperatures ( $90^\circ\text{C}$ .) increases again; (3) it increases with the amount of water present up to a certain point and then decreases, the optimum amount varying with the soil; and finally (4) it is greatly influenced by the state of division of the soil and aeration. Sterile soil produces small amounts of carbon dioxide, but, when rein-

<sup>32</sup> Kissling, R. and Fleischer, M. Landw. Jahrb., 20: 876-889. 1891.

<sup>33</sup> Dehérain, P. P. and Demoussy, E. Ann. Agron., 22: 305-337. 1896.

oculated with soil extract, it forms twenty-five times as much  $\text{CO}_2$ .<sup>34</sup> Sterilized and inoculated soil gives two to five times as much carbon dioxide as unsterilized soil.

When manure is treated with disinfectants (thymol, phenol,  $\text{HgCl}_2$ ), little  $\text{CO}_2$  is produced; this proves that the process of decomposition of organic matter in the rotting of manure and the evolution of carbon dioxide are biological in nature. Since microorganisms are influenced in their activities by the temperature and moisture content of the medium, one would expect that a change in temperature and moisture within certain limits, would modify the evolution of  $\text{CO}_2$ . Decomposition of the organic matter was found to vary roughly with the amount of oxygen available, although some  $\text{CO}_2$  is formed in the complete absence of oxygen.<sup>20</sup> Under anaerobic conditions, the organic matter is not decomposed completely and a large part of the energy is left in the form of intermediary products; the amount of  $\text{CO}_2$  liberated is small and cannot serve as good an index of decomposition as the evolution of gas under aerobic conditions. When the amount of oxygen absorbed was used as an index of oxidation in soil, the rate of absorption was found to increase with the temperature, the amount of water (up to a certain point), and the amount of calcium carbonate; oxygen absorption was favored by conditions obtaining in the surface soil as opposed to those in the subsoil.<sup>35</sup>

When a soil is air-dried and then moistened, or when it is partially sterilized by means of heat or chemicals, there follows a decided increase in microbiological activities, resulting in the liberation of greater amounts of  $\text{CO}_2$ . When undecomposed organic matter is added to the soil, it is decomposed with a rapidity depending not only on the nature of the substance added, but also upon the presence of available nitrogen, the soil reaction, moisture, aeration, etc. The amount of  $\text{CO}_2$  produced from the decomposition of a certain organic substance depends also upon the nature of the organisms which are concerned in the process; different organisms attack the same substance, but yield different products.

The nature and composition of the organic material added to the soil greatly influence the type of organism developing and the mechanism of the process of decomposition. Sugars decompose in the soil very rapidly. The absence of available nitrogen does not become a limiting factor, since nitrogen-fixing organisms can use sugars as sources

<sup>34</sup> Sewerin, S. A. *Centrbl. Bakt.* II, 13: 616; 28: 561. 1910; 32: 498. 1912.

<sup>35</sup> Wollny, 1897 (p. 417); Russell, E. J. *Jour. Agr. Sci.*, 1: 261-279. 1905.

of energy and develop readily when large amounts are added to the soil. One per cent of glucose added to soil disappears within forty-eight hours. The decomposition of cellulose, however, is carried out by certain specific bacteria and fungi, which require an available source of nitrogen; the amount of the latter in the soil will, therefore, become the limiting factor in the decomposition of cellulose. In the case of organic substances of plant origin, the monosaccharides and starches are decomposed first, followed by the pentosans, cellulose, pectins, and proteins; the strongly resistant carbonaceous residue (consisting largely of lignin and waxes) is decomposed only very slowly.<sup>36</sup> Organic matter containing sufficient nitrogen, like ground leguminous plants, cottonseed meal, dried blood, and fungus protoplasm, decomposes more rapidly; the nitrogen, sooner or later, depending on its concentration, becomes liberated as ammonia. Substances rich in proteins decompose at an entirely different rate than those composed principally of carbohydrates. Materials rich in oxygen and low in carbon decompose more quickly than those rich in carbon and poor in oxygen.<sup>37</sup> The following amounts of various organic substances, on a per cent basis, were decomposed in 21 days, as indicated by the evolution of carbon dioxide, using 10 grams of material per 100 grams of soil:

	<i>per cent decomposed</i>
Clover.....	59.7
Glucose.....	42.1
Rice straw.....	29.0
Oak leaves.....	17.7
Wheat straw.....	14.5
Cellulose.....	11.8

The addition of available nitrogen ( $\text{NaNO}_3$ ) to a nitrogen poor substance, like straw, stimulates its decomposition but not that of a substance containing sufficient nitrogen, like alfalfa meal. This is brought out in Fig. 48. The lower the nitrogen content of the plant material, the greater is the effect of additional inorganic nitrogen upon the rapidity of its decomposition. A high lignin content, however, will interfere with the favorable effect of the additional nitrogen, as shown in the case of the oak leaves. Analyses of these materials were reported in Table 66.

These results confirm the earlier observations that the  $\text{CO}_2$  content of the soil rises and falls with the amount of organic matter present and

<sup>36</sup> Van Suchtelen, F. H. H. *Centrbl. Bakt.* II, 28: 45-89. 1910.

<sup>37</sup> Dvorak, 1912 (p. 492); Starkey, R. L. *Soil Sci.*, 17: 293-314. 1924.

that the addition of manure to the soil stimulates the evolution of carbon dioxide. Fresh manure stimulates this evolution more than old decomposed manure, because of the introduction of both available energy and available nitrogen. Grinding of peat increases its rate of decomposition.

In the study of decomposition of leaves and needles of different trees, a correlation was found<sup>38</sup> to exist between the chemical composition and rate of decomposition in the case of one species, but not when different species of plants were compared. These results however, need further elucidation.

It was suggested<sup>39</sup> that CO<sub>2</sub> formation in soil, whether treated with organic matter or not, does not proceed in accordance with the growth law of bacteria. Under aerobic and constant environmental conditions, it proceeds in accordance with the equation

$$x = a k t^m$$

In which  $x$  is the quantity of CO<sub>2</sub> produced in time  $t$ ,  $a$  is the initial CO<sub>2</sub> content of the soil, and  $k$  and  $m$  are constants. However, experiments with peat and other soils have shown that the CO<sub>2</sub> formation in the soil is not always necessarily parallel to the concentration of carbon in the soil.

As to the method used for measuring the evolution of carbon dioxide, at first the air freed from CO<sub>2</sub> was passed through the soil placed in a container, and the CO<sub>2</sub> in the outgoing air measured.<sup>40</sup> This method was later modified so that air, previously freed from CO<sub>2</sub>, was passed continuously over the surface of the soil in closed containers. Under these conditions the soil more nearly approaches normal, since constant aeration through the soil greatly accelerates microbiological activities.<sup>41</sup>

<sup>38</sup> Melin, E. Ecology, 11: 72-101. 1930.

<sup>39</sup> Lemmermann, O. and Weissmann, H. Ztschr. Pflanzenern. Düng., 2B: 387-395. 1924.

<sup>40</sup> Pettenkofer, M. Chem. Soc. Trans., 10: 292. 1858; Peterson, P. Landw. Vers. Sta., 13: 155-175. 1870; Wollny, 1897 (p. 417); Stoklasa and Ernest, 1905 (p. 31); Lemmermann, O., Fischer, H. and Fresenius, L. Landw. Jahrb., 41: 217-256. 1911; Klein, M. A. Jour. Amer. Soc. Agr., 7: 49. 1915; Gainey, P. L. Soil Sci., 7: 293-311. 1919.

<sup>41</sup> Fred, E. B. and Hart, E. B. Wis. Agr. Exp. Sta. Res. Bul. 35. 1915; Fraps, G. S. Texas Agr. Exp. Sta. Bul. 181. 1915; Potter, R. S. and Snyder, R. S. Iowa Agr. Exp. Sta. Res. Bul. 39. 1916; Merkle, F. G. Jour. Amer. Soc. Agron., 10: 281-302. 1918; Neller, J. R. Soil Sci., 5: 225-239. 1918; Waksman and Starkey, 1923 (p. 717).

*Formation of ammonia (and nitrate) as an index of decomposition of organic matter in the soil.* Nitrogen is present in the complex organic substances largely in the form of proteins. In the processes of decomposition, these are hydrolyzed through a series of intermediary complexes, with the final liberation of the nitrogen as ammonia; this is oxidized in normal aerated soil to nitrites, and then to nitrates.<sup>42</sup> The determination of the ammonia and nitrate thus produced can serve as a good index of the course of decomposition of simple and complex organic nitrogenous compounds in the soil. In the case of some soils rich in organic matter, as in the fertile prairie soils, the transformation of the ammonia into nitrate goes on less rapidly than the formation of ammonia, hence the latter may accumulate.<sup>43</sup> In the presence of carbohydrates or other carbon compounds, serving as sources of energy for microorganisms, the ammonia and nitrate are rapidly changed again to proteins; as a result of this, the amount of mineralized nitrogen is no true index of decomposition. The production of ammonia and nitrate can serve as an index of the availability of nitrogen in organic fertilizers, based on the fact that different organic substances decompose with different rapidity.<sup>44</sup>

Attempts have been made (p. 693) to differentiate fertility of different soils, using the rapidity of decomposition of organic nitrogenous compounds, either in solution or in soil.<sup>45</sup> The results were frequently difficult to interpret. The differences obtained between the formation of ammonia in different soils were found to be due to a number of causes other than the microbial flora of the soils.<sup>46</sup> This becomes self-evident when we consider the following factors: (1) a large number of soil organisms are capable of decomposing proteins with the formation of ammonia; (2) the amount of ammonia formed depends also upon the nature of the carbon of the organic matter added to the soil in the form of nitrogenous and non-nitrogenous substances; (3) a part of the am-

<sup>42</sup> A detailed study of the decomposition of nitrogenous substances in the soil is given by Lathrop, 1917 (p. 415); in forest soil by Süchting, H. *Ztschr. Pflanzernähr. Düngung.*, 1: 113-154. 1922.

<sup>43</sup> Wyatt, F. A., Ward, A. S. and Newton, J. D. *Sci. Agr.*, 7: 1-24. 1926.

<sup>44</sup> Lipman, J. G. *Jour. Indus. Engin. Chem.*, 2: 146-148. 1910; Löhnis, F. and Green, H. H. *Centrbl. Bakt. II*, 40: 52-60. 1914.

<sup>45</sup> Remy, 1902 (p. 693); Fischer, H. *Centrbl. Bakt. II*, 24: 62-74. 1909; *Landw. Jahrb.*, 41: 755-822. 1911; Löhnis, F. *Centrbl. Bakt. II*, 12: 262-267, 448-463. 1904; 14: 1-9. 1905; Vogel. *Centrbl. Bakt. II*, 27: 593-605. 1910; Lipman, J. G. *N. J. Agr. Exp. Sta. 27th Ann. Rpt.* 1906, 119-187.

<sup>46</sup> Temple, J. C. *Ga. Agr. Exp. Sta. Bul.* 126. 1919.

monia changes into nitrates, depending upon the physico-chemical, chemical and biological condition of the soil; (4) the amount of ammonia that a soil can hold depends upon its initial reaction, nature of absorbed bases and buffer content.

According to some investigators,<sup>47</sup> no necessary fundamental difference exists between bacteriological processes in soil and solution media. Among the important factors affecting the transformations in soil and in solution are the nature, quantity, and distribution of substrate, aeration, diffusion, absorption, destruction or evaporation of metabolic products; reaction of the medium, temperature and duration of experiment are all of importance. Ammonia accumulation can be used effectively as an index of the activities of pure cultures of microorganisms or of the complex soil flora upon nitrogenous organic materials.

The curves of ammonia accumulation and of CO<sub>2</sub> production from the same nitrogenous organic substance added to the soil were found<sup>48</sup> to run parallel, tending to indicate that both can be taken as indices of the rapidity of decomposition of highly nitrogenous organic substances in the soil. However, when the curves of NH<sub>3</sub> accumulation from dried blood and CO<sub>2</sub> evolution from another organic substance, like soy bean meal, were compared, even in the same soil, no parallelism at all was observed.<sup>49</sup> This is due to the fact that different organic substances are attacked at different rates even by the same groups of microorganisms. The addition of these different organic substances to the soil may stimulate the development of different groups of organisms, with the result that there is no basis for comparison of the decomposition of two substances, even in the same soil, when two different indices of decomposition of organic matter (CO<sub>2</sub> and NH<sub>3</sub> evolution) are employed. It was found<sup>50</sup> that, for a certain amount of organic nitrogen, in the form of soy bean cake and herring cake, changed to ammonia, twice as much organic matter is changed to CO<sub>2</sub> in an acid soil as in a loam soil. By comparing two sources of organic matter, it was found that 1.5 times as much is changed to CO<sub>2</sub> in herring cake as in soy bean cake. The mechanical soil conditions influence also markedly the relation between ammonia, nitrate and carbon dioxide production.<sup>51</sup>

<sup>47</sup> Löhnis, F. and Green, H. H. *Centrbl. Bakt.* II, 40: 457-479. 1914.

<sup>48</sup> Gainey, 1919 (p. 610).

<sup>49</sup> Neller, 1918 (p. 610).

<sup>50</sup> Miyake, K. and Nakamura, K. *Jour. Biochem. Tokyo.*, 3: 27-54. 1923.

<sup>51</sup> Carpenter, P. H. and Rose, A. K. *Indian Tea Assoc. Sci. Dept. Quart. Jour.* 1921, p. 103.

*Influence of plant residues upon the growth of cultivated plants.* A knowledge of the chemical composition of plant residues added to the soil and of their decomposition by microorganisms is essential for our understanding of the influence of straw and other plant residues upon plant growth. Higher cultivated plants may have to compete with microorganisms for the available plant food, especially the nitrogen. When large amounts of green manure or straw are plowed under and a crop is planted soon afterwards, distinct injury to the crop may set in. This injury is largely a phenomenon of nitrogen starvation. Krüger and Schneidewind<sup>52</sup> submitted definite evidence that the addition of cellulose to the soil stimulates the development of various soil organisms which leads to the disappearance of soil nitrates and prevents the plants from obtaining sufficient nitrogen for their growth. The fact that leguminous plants grow readily in soil in the presence of materials rich in cellulose, since they are independent of the soil nitrogen, and the fact that no ill effects were observed in partially sterilized soil served to prove the above theory. Pfeiffer and Lemmermann<sup>53</sup> pointed out that the injurious effect of straw upon plant growth was due not to denitrification but to the development of organisms which assimilated the nitrates and used the nitrogen for the synthesis of microbial protoplasm. It was later found that substances like hay and sugar cause a harmful effect upon plant growth the first year and a beneficial effect the second year; i.e. the application of materials rich in carbohydrate depressed crop growth when applied just previous to planting but stimulated crop growth when a considerable period elapsed.<sup>54</sup> According to Rahn,<sup>55</sup> easily assimilable carbon compounds are present in the soil only in minute quantities; ammonia and nitrate can, therefore, accumulate as a result of a gradual decomposition of the soil organic matter. The addition of straw and other plant substances rich in available carbon (energy) brings about an increase in the development of microorganisms as a result of which a nitrogen minimum may occur. This will last as long as easily decomposable non-nitrogenous organic compounds are

<sup>52</sup> Krüger, W. and Schneidewind, W. *Landw. Jahrb.*, **28**: 217-252. 1899; **30**: 633-648. 1901.

<sup>53</sup> Pfeiffer, Th. and Lemmermann, O. *Landw. Vers. Sta.*, **54**: 386-462. 1900.

<sup>54</sup> Bredemann, G. *Landw. Jahrb.*, **43**: 669-694. 1912; Hutchinson, H. B. *Jour. Agr. Sci.*, **9**: 92-111. 1918; Albrecht, W. A. *Soil Sci.*, **14**: 299-305. 1922; **20**: 253-265; Murray, T. J. *Soil Sci.*, **12**: 233-260. 1921; Scott, H. *Jour. Amer. Soc. Agron.*, **13**: 233-258. 1921; Martin, T. L. *Soil Sci.*, **20**: 159-164. 1925; May, F. V. *Mitt. Wien. Hochsch. Bodenk.*, **2**: 433-454. 1913-1914.

<sup>55</sup> Rahn, O. *Ztschr. Tech. Biol.*, **7**: 172-186. 1919.



still left. The nitrogen minimum appears more quickly and lasts longer in nitrogen-poor than in nitrogen-rich soils. During this condition, the plants cannot obtain any nitrogen from the soil. The addition of available nitrogen overcomes the harmful results.

Studies<sup>56</sup> on the nutrition of seedlings, on the influence of manure upon cellulose decomposition<sup>57</sup> and on the influence of wood products upon the growth of leguminous and non-leguminous plants (table 72)<sup>58</sup> definitely indicate that we are not dealing here with an injury to the process of nitrification but with the assimilation of nitrate by the soil fungi and bacteria that use the cellulose as a source of energy. Collison and Conn<sup>59</sup> concluded that two separate harmful factors are associated with the influence of straw and other plant residues upon plant growth: (1) a toxic chemical agent which acts upon the plants immediately after

TABLE 72

*Influence of different kinds of wood on the growth of oats and red clover*

TREATMENT	YIELD OF OATS, AVERAGE	YIELD OF RED CLOVER, AVERAGE	
		Uninoculated	Inoculated
	<i>grams</i>	<i>grams</i>	<i>grams</i>
None.....	26.6	27.5	44.0
1.5 per cent coarse wood.....	18.3	26.5	37.3
3.0 per cent coarse wood.....	14.8	21.5	43.0
3.0 per cent fine wood.....	21.0	24.5	41.5
3.0 per cent wood burned and ash used.....	27.5	60.0	56.0

germination, the effect not being pronounced in soils; (2) a biological factor as a result of the competition between soil microorganisms and plants for the available nitrogen.

It has also been suggested<sup>60</sup> that the roots of the growing plants liberate organic matter much of which is non-nitrogenous; this favors the development of nitrate-consuming organisms in the soil, with the subse-

<sup>56</sup> Kellerman, K. F. and Wright, R. C. Jour. Agr. Res., 2: 101-113. 1914.

<sup>57</sup> Barthel, C. and Bengtsson, N. Soil Sci., 18: 185-200. 1924; Ibid., 1926 (p. 388); Anderson, 1926 (p. 390).

<sup>58</sup> Viljoen, J. A. and Fred, E. B. Soil Sci., 17: 199-208. 1924.

<sup>59</sup> Collison, R. C. and Conn, H. J. N. Y. (Geneva) Agr. Exp. Sta. Tech. Bul. 114. 1925.

<sup>60</sup> Lyon, T. L., Bizzell, J. A. and Wilson, B. D. Jour. Amer. Soc. Agron., 15: 457-466. 1923.

quent transformation of the nitrates into other nitrogenous substances. The composition of the organic matter liberated (or made available) by the living and decomposing plant roots has a potent influence upon the activity of the nitrate consuming organisms. The amounts of nitrate nitrogen recovered by leaching, when different roots and dried blood were added to the soil, were found to vary directly with the percentages of nitrogen in these substances, by comparing the decomposition of organic matter added, in quantities sufficient to contain 0.6 gram of nitrogen, to 28 pounds of soil and incubating three months (table 73). There was about the same amount of nitrate formed in the control soil and in the soil to which clover was added. About 1.7 per cent of nitrogen was sufficient to allow the microorganisms to utilize the available energy in the fresh organic matter; the greater the amount of organic

TABLE 73

*Relation of nitrogen content of various plant roots to nitrogen in leachings from soil in which they were decomposed*

MATERIAL USED	NITROGEN	WEIGHT OF MATERIAL ADDED	TOTAL NITRATE NITROGEN IN LEACHINGS
	<i>per cent</i>	<i>grams</i>	<i>mgm.</i>
Control soil.....	....	....	946.6
Oat roots.....	0.45	133.3	207.3
Timothy roots.....	0.62	96.8	398.4
Maize roots.....	0.79	75.9	510.6
Clover roots.....	1.71	35.1	924.4
Dried blood.....	10.71	5.6	1751.1

matter added, the less is the amount of nitrate found and the greater was the amount used up by the microorganisms. In soil treated with dried blood (any other source of organic matter containing more than 1.7 per cent nitrogen could have been used), more nitrate was found than in the control soil. The actual amount of nitrate produced was regulated by the per cent of nitrogen contained in the organic matter. The higher the nitrogen content, all other factors being alike, the greater will be the amount of nitrate formed. Similar results were obtained when the actual consumption of nitrogen by microorganisms bringing about the decomposition of different plant materials was measured.<sup>61</sup>

The nitrogen thus assimilated by the microorganisms and synthesized into cell substance will sooner or later become available, when these cells

<sup>61</sup> Waksman, S. A. and Tenney, F. G. *Soil Sci.*, **24**: 317-333. 1927.

begin to decompose, as seen in table 74.<sup>62</sup> When the decomposition of two plant materials, one of a low nitrogen content like timothy residues and one of a high nitrogen content like clover residues was compared, that of the former was found to be extended over a longer period of time. The more rapid decomposition of the clover residues is accompanied by a more rapid increase in the number of microorganisms concerned in the process later followed by a more rapid drop. The removal of nitrate from the soil solution will, therefore, not be as prolonged in the case of clover as in the case of timothy residues; this tends to explain the smaller depression of the nitrate content of the soil following addition of clover or other leguminous materials than that following the addition of timothy or other plant residues low in nitrogen.<sup>63</sup>

TABLE 74  
*Milligrams of nitrate nitrogen per 100 grams of dry soil*

ORGANIC MATTER ADDED (0.3-0.6 PER CENT)	AT START	AFTER, WEEKS					
		12	20	24	28	32	40
None.....	0.59	0.74	0.55	1.25	1.50	3.51	2.65
Paper.....	0.59	0	0	Trace	Trace	0.20	1.29
Straw.....	0.59	0	0	1.81	2.20	3.18	3.70
Clover.....	0.59	1.22	2.01	2.40	2.00	3.62	3.82

*Summary.* In the decomposition of plant and animal residues various chemical constituents are attacked at a different rate, by different organisms, depending upon the nature of the organic matter, microorganisms concerned and environmental conditions. The sugars and other water-soluble substances as well as the starches, proteins, and their derivatives are attacked very rapidly by a number of bacteria, fungi and other organisms. These are followed soon by the pentosans and other hemicelluloses, as well as the cellulose; the latter is decomposed only by a number of specific organisms, found among the bacteria, fungi, and actinomyces. The lignin is most resistant to decomposition, especially under anaerobic conditions.

All microorganisms require a certain amount of energy for the building up of their protoplasm as well as a certain minimum of nitrogen, phosphorus and other minerals. In the case of heterotrophic, non-

<sup>62</sup> Hill, H. H. Virginia Agr. Exp. Sta. Tech. Bul. 6. 1915.

<sup>63</sup> Wilson, B. D. and Wilson, J. K. N. Y. (Cornell) Agr. Exp. Sta., Mem. 95. 1925.

nitrogen-fixing microorganisms, the energy is obtained either from nitrogen-free organic compounds or from the proteins and their derivatives. The nitrogen is obtained from inorganic nitrogenous salts, such as ammonium compounds and nitrates, or from complex organic compounds, such as proteins and their derivatives. Phosphates and other minerals are obtained from the inorganic or organic compounds present.

When an organism has to derive both its carbon and nitrogen from proteins, only a small part of the nitrogen is reassimilated, while a larger part will remain as a waste product (ammonia). Several factors contribute to this phenomenon:

1. Only 10 to 40 per cent of the carbon is reassimilated by the organism and synthesized into protoplasm; a larger part is given off as  $\text{CO}_2$ , in the process of energy utilization, or is left in the form of undecomposed material or in the form of intermediary products. The smaller the amount of carbon assimilated by the organism, the less is the amount of protoplasm synthesized and, therefore, the less is the amount of nitrogen assimilated and the greater is the amount left in the medium as a waste product, largely  $\text{NH}_3$ .

2. The microbial protoplasm may contain a lower per cent of nitrogen than the original protein. This will tend further to diminish the amount of reassimilated nitrogen. The excess nitrogen will be liberated as ammonia or left in the form of various protein degradation products.

In the presence of available carbohydrates, however, the microorganisms assimilate the available ammonia nitrogen and convert it into microbial protoplasm. The greater the quantity of carbohydrate present for a given amount of protein or its derivatives the greater is the amount of nitrogen that will be reassimilated by microorganisms and the smaller the remainder left as ammonia. The larger the ratio of the protein-free substances to the protein, the smaller is the amount of ammonia liberated. This has an important bearing upon the liberation of ammonia in the soil, since the great mass of organic matter usually added to the soil, in the form of manures and plant residues, contains a low percentage of nitrogen and a high percentage of energy-yielding material. The available nitrogenous plant food in the soil is also greatly affected by the conditions under which decomposition takes place, since, under different conditions, different organisms take part in the processes and, therefore, bring about different sets of reactions.

The organic substances of plant and animal origin undergoing decomposition, comprising the more resistant chemical complexes, largely

lignins and certain hemicelluloses, the incompletely decomposed cellulose, fats and certain pentosans, and those organic complexes which have been synthesized by the microorganisms bringing about the decomposition, including organic nitrogenous complexes and certain carbohydrates, make up the soil humus. This humus plays a very important physical and chemical rôle in the soil. It is also used as a nutrient by certain groups of microorganisms, although in general it is much more resistant to decomposition than fresh plant and animal residues. It is characterized by a carbon-nitrogen ratio which tends to approach 10. It is very complex in composition, depending upon the plant materials from which it originated, upon the microorganisms bringing about its decomposition and upon the environmental conditions under which decomposition is taking place.

## CHAPTER XXV

### DECOMPOSITION OF ORGANIC MATTER IN STABLE MANURES AND ARTIFICIAL MANURES, AND MICROORGANISMS CONCERNED

*Chemical composition of stable manure and its importance in the soil.* Stable manure consists of solid animal excreta, of urine and of straw; the fresh manure ordinarily contains 20 to 30 per cent of dry matter. The chemical composition of the manure varies with the nature of the animal, system and kind of feeding and type of bedding used. A ton of farm-yard manure is reported<sup>1</sup> to contain 9.8 pounds of nitrogen, 2.82 pounds of phosphorus and 7.14 pounds of potassium; the respective quantities for sheep manure were found to be 28.0, 4.4 and 20.0; for horse manure, 14.0, 2.2, and 13.0; for dairy cows, 11.0, 1.9 and 10.0; for steers, 13.5, 2.4 and 11.0. On a dry basis, manure usually contains about 1.9 to 2.8 per cent nitrogen, 0.7 to 1.2 per cent phosphoric acid, and 0.5 to 3.0 per cent potassium. The abundance of the most important nutrients in different manures is given in table 75.

Nitrogen is usually considered to be the most valuable element in the manure. Out of every 100 parts of nitrogen consumed in the feed, different animals were found to excrete the following relative amounts of this element:

NATURE OF ANIMAL	COW	OX	HORSE
Per cent of the nitrogen consumed:			
Found in the solid matter.....	47.5	33.9	32.4
Found in the urine.....	31.0	54.8	60.7

These results show that, in the case of mature animals, 78.5 to 93 per cent of the nitrogen in the feed is again excreted by the animal in the solid and liquid portions of the manure. About half of the nitrogen found in fresh manure is in the form of ammonia and urea, while the other half is in the form of proteins and other complex organic nitrogenous compounds.

<sup>1</sup> Ames, J. W. Penn. Agr. Exp. Sta. Bull., 175. 1920; Ruschmann, G. Handb. Pflanz. Düng. by Honcamp. J. Springer, Berlin, 2: 162-234. 1931; Mangold, E. Handbuch der Ernährung und des Stoffwechsels der landwirtschaftlichen Nutztiere. J. Springer, Berlin., 2, 1929.

The dry portion of the manure is rich in cellulose, pentosans, lignin, nitrogenous compounds and minerals. The organic matter undergoes a series of transformations by microorganisms, both in the compost heap and in soil, with a rapid reduction in the cellulose and pentosan content, and a relative increase in the lignin and in the proteins, with the result that the organic nitrogen becomes available only after a series of transformations.

The importance of stable manure in soil processes has been variously ascribed to four distinct factors: 1. Manure offers a readily available supply of nitrogen, phosphoric acid and potash for the growth of higher plants. 2. Since manure undergoes rapid decomposition in the soil, it is a good source of carbon dioxide, which is necessary for plant growth.

TABLE 75  
*Chemical composition of stable manures\**  
On per cent basis of total material

	TOTAL ORGANIC MATTER	N	P <sub>2</sub> O <sub>5</sub>	K	Ca
Milk cow.....	20.0	0.42	0.25	0.50	0.45
Horse.....	25.4	0.58	0.28	0.53	0.25
Sheep.....	30.0	0.85	0.23	0.67	0.33
Pig.....	25.0	0.45	0.19	0.60	0.08
Fresh manure.....	21.0	0.45	0.27	0.55	0.56
Rotted manure.....	17.0	0.50	0.34	0.55	0.70

\* Stutzer, A. and Honcamp, F.: *Die Behandlung und Anwendung von Stall-dünger und Jauche*. P. Parey. Berlin. 1928.

3. The organic matter of the manure serves the purpose of replenishing the supply of humus in the soil. 4. Manure exerts a favorable influence upon the soil microbiological activities; in many instances claims were put forth that the importance of stable manure in soil productivity is due to its bacterial content, and, therefore, to the introduction of large numbers of bacteria into the soil; this claim may be regarded, however, as unjustified, as shown elsewhere (746).

*Decomposition of stable manure.* Thirty to forty per cent of the dry weight of stable manure consists of cellulose and twenty to thirty per cent of pentosan.<sup>2,3</sup> When manure undergoes decomposition, whether

<sup>2</sup> Stoklasa, J. *Ztschr. landw. Versuchsta.* Oester., 10: 440. 1907; Fühling's *landw. Ztg.*, 56: 411. 1907.

<sup>3</sup> Waksman, S. A. and Diehm, R. A. *Jour. Amer. Soc. Agron.*, 21: 795-809. 1929.

in the compost heap or in the field, the cellulose and the pentosans disappear rapidly. Hébert<sup>4</sup> reported in 1892 that, in the "fermentation" of sheep manure, the water-soluble and fatty substances, as well as the "straw gum" (pentosan) are the first to disappear; these are followed by the cellulose. A highly carbonaceous substance, described as "vasculose," which is practically the same complex now better known as lignin, appears in large proportion in the manure. This "vasculose" is present in the original plant material; it is only partly decomposed and variously modified during the process of decomposition of the manure, a large part of it remaining in the residual compost, as shown in table 76. Dehérain<sup>5</sup> believed that the rôle of litter in manure consists

TABLE 76

*Decomposition of straw, in the presence of inorganic nutrients, under aerobic and anaerobic conditions (Hébert)*

CHEMICAL COMPLEXES	STRAW AT START	COMPOSTED STRAW	
		Aerobic	Anaerobic
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
NH <sub>4</sub> -N.....	2.64	0.40	1.40
Organic N.....	0.39	1.20	1.48
Ether-soluble substances.....	0.46	0.30	0.29
Water-soluble substances.....	1.53	0.26	0.15
Cellulose.....	14.12	6.18	5.98
Straw gum (xylan).....	10.00	4.67	3.97
Vasculose (lignin).....	14.01	11.75	8.91
Ash.....	6.32 (3.57 + 2.75*)	6.40	5.56

\* The 2.75 per cent ash were added in the form of mineral nutrients.

not only in the absorption of the liquids excreted by the animals and in supplying a soft bed for them, but also in supplying "vasculose," which characterizes the manure and gives to it its special fertilizing value.

Dehérain distinguished two processes of manure decomposition: (1) an aerobic process, when the temperature of the compost goes up to 65°–70°C.; (2) an anaerobic process, with a maximum temperature of 30° to 35°C. The composition of the gases given off in the decomposition of manure is given in table 77. A large part of the cellulose of the straw

<sup>4</sup> Hébert, A. *Compt. Rend. Acad. Sci.*, 110: 969–972; 115: 1321–1323. 1892; *Ann. Agron.*, 18: 536–550. 1892; *Exp. Sta. Record*, 5: 141–158. 1895.

<sup>5</sup> Dehérain, P. P. *Ann. Agron.*, 10: 385–409. 1886; 14: 97–133. 1888; 1902 (p. 767); See also Gayon, U. *Compt. Rend. Acad. Sci.*, 98: 528–531. 1889.



was believed to be decomposed, with the formation of  $\text{CH}_4$  and  $\text{CO}_2$ . The residue left, after a part of the nitrogenous substances and the cellulose have undergone decomposition, forms the black matter of the manure. This is accompanied by the transformation of the ammonia and urea nitrogen into organic compounds.

In studies on the digestion by sheep of grass hay and clover hay, harvested at different stages of growth, König<sup>6</sup> found that the higher the lignin and cutin content of the plant materials, the less is their digestibility. Cellulose and pentosans were found to be more readily digested by the animals than lignin. According to a more recent contribution by Rubner,<sup>7</sup> cellulose of different origin differs markedly in its digestibility; lignin itself was digested only to a limited extent in the animal organism, but to a considerably less extent than the cellulose and the pentosans.

TABLE 77  
*Composition of gases in different parts of the manure heap*

	TOP LAYER OF HEAP	MIDDLE LAYER OF HEAP	BOTTOM LAYER OF HEAP
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Carbon dioxide.....	21.6	31.0	37.1
Oxygen.....	0.0	0.0	0.0
Methane.....	0.0	33.3	58.0
Nitrogen.....	78.4	35.5	4.9

König has further shown<sup>8</sup> that the decomposition of stable manure involves processes of oxidation of the carbon compounds, so that, at the end of the first year, following application of the manure to the soil, seventy-five per cent of the carbon has disappeared; decomposition is more rapid during the warmer periods of the year than during the colder. The pentosans are decomposed more rapidly than the total organic matter, while the lignin disappears more slowly; decomposition of manure was thus found to be accompanied by a gradual enrichment in the lignin, as measured by the increase in the methoxyl content. Both the total and easily soluble nitrogen compounds in the manure diminished

<sup>6</sup> König, J. Fürstenberg, A. and Murdfield, R. Landw. Vers. Sta., **65**: 55-110. 1907.

<sup>7</sup> Rubner, M. Die Naturwiss., **16**: 1011-1019. 1928.

<sup>8</sup> König, J., Bach, M., Balks, R. and Hasenbäumer, J. Mitt. Deut. landw. Gesell. Nos. 26, 27. 1926; Landw. Vers. Sta., **104**: 245-284. 1926; See also Sauerlandt, W. Wiss. Arch. Landw. A., **2**: 434-471. 1921.

rapidly after its application to the soil; this was believed to be due either to denitrification or to a removal of the nitrogen to the subsoil. Only about one-third of the nitrogen and of the phosphoric acid and seventy per cent of the potash in the manure were made available to the

TABLE 78  
*Chemical composition of various fresh manures, litter free*  
On per cent basis of dry material

CHEMICAL CONSTITUENT	SHEEP MANURE*	HORSE MANURE†	COW MANURE*
Ether-soluble substances.....	2.83	1.89	2.77
Cold water-soluble organic matter.....	19.19	3.19	5.02
Hot water-soluble organic matter.....	5.73	2.39	5.32
Hemicelluloses.....	18.46	23.52	18.57
Cellulose.....	18.72	27.46	25.23
Lignin.....	20.68	14.23	20.21
Total nitrogen.....	4.08	1.09	2.38
Ash.....	17.21	9.11	12.95

\*Solid and liquid excreta.

†Solid excreta only.

TABLE 79  
*Composition of horse manure at different stages of decomposition*  
On per cent basis of dry material

	FRESH	39 DAYS	96 DAYS	157 DAYS	290 DAYS
Ether-soluble fraction.....	1.89	1.89	1.47	0.88	0.95
Cold water-soluble fraction.....	3.19	4.11	4.73	4.36	3.81
Hot water-soluble fraction.....	2.39	3.86	3.37	2.19	1.90
Hemicelluloses.....	23.52	22.84	15.76	13.36	12.67
Cellulose.....	27.46	23.18	16.07	6.98	5.97
Lignin.....	14.23	16.63	17.92	20.54	28.43
Crude protein.....	6.81	7.00	14.81	18.56	16.38
Ash.....	9.11	13.64	20.93	22.22	19.32

growing crops during the first two years after the application of the manure.

These results were later confirmed and further extended,<sup>9</sup> as shown in tables 78 and 79. All the constituents of the manure undergo decomposition by various groups of microorganisms. The residual compost consists largely of lignin and modified lignin complexes, which are

<sup>9</sup> Waksman and Diehm, 1929 (p. 620).

resistant to decomposition, of proteins synthesized largely by microorganisms, of small amounts of cellulose and hemicelluloses undergoing decomposition, of mineral constituents and of other substances in lower

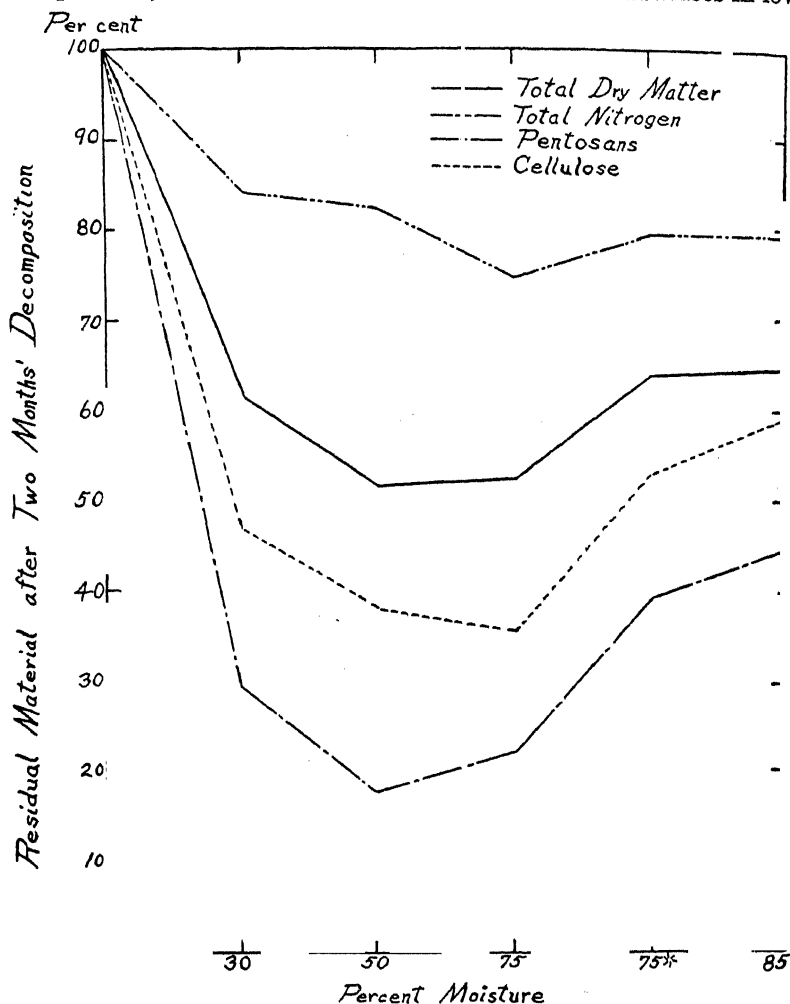


FIG. 55. The influence of moisture and aeration upon the decomposition of fresh horse manure (from Egorov).

concentrations. It is interesting to note that, while in the fresh manure the cellulose is considerably in excess over the hemicelluloses, in composted manure the latter are in excess over the cellulose. This is due

to the fact that some of the hemicelluloses, especially the pentosans, are more readily decomposed than the cellulose; however, other hemicelluloses, such as galactans and certain uronic acid complexes, are more resistant to decomposition, hence they accumulate; in addition to that, considerable quantities of hemicellulose (mycodextrans, mycogalactans, bacterial gums) are synthesized by the microorganisms bringing about the decomposition of the manure.

Falck<sup>10</sup> reported that in certain processes of composting of manure, especially when it has reached the stage of nitrate formation, the lignin is also attacked by various bacteria and actinomyces, similar to the process of "corrosion" in wood carried out by certain higher fungi (p. 401). The influence of moisture and aeration upon the rapidity and nature of decomposition of several of the most important organic complexes of the manure, during the process of composting of fresh horse manure, is brought out in fig. 55.<sup>11</sup>

*Nitrogen transformation in the decomposition of manure.* Most of the nitrogen consumed by animals is excreted again in the solid and liquid portions of the manure, as shown by Gay and Dupont<sup>12</sup> (table 80). At least a half of the nitrogen, and frequently more, of the manure is found in the urine, as urea and ammonium carbonate. This part of the nitrogen nitrifies very readily, as soon as the manure is added to the soil. Even the presence of straw does not reduce the rapidity of the process of nitrate formation. These results were confirmed by other investigators,<sup>13</sup> who reported that 18 to 50 per cent of the nitrogen in manure is changed to nitrate in six to twelve weeks, the process being best where the urine is added to the manure. The nitrogen in the solid excreta does not change readily into nitrates; it may take place, however, only after the manure has been composted for a considerable period of time.<sup>14</sup>

Barthel and Bengtsson<sup>15</sup> freed stable manure from the ammonia nitrogen present in it, by distillation with or without a vacuum; when manure thus prepared was added to various soils, no nitrate formation took place, showing that only the ammoniacal and urea nitrogen in the manure undergo active nitrification, but not the nitrogen present in the

<sup>10</sup> Falck, R. *Cellulosechemie*, 9: 1-6. 1928.

<sup>11</sup> Egorov, M. A. *Ann. Inst. Agron. Moscow*, 17 (1911): 1-58. 1912.

<sup>12</sup> Dehérain, P. P. 1902 (p. 767).

<sup>13</sup> Wagner, P. *Arb. deut. landw. Gesell.*, 80: 1-335. 1903; Löhnis, F. and Smith, J. H. *Fühling's landw. Ztg.*, 63: 153-167. 1914.

<sup>14</sup> Joshi, N. V. *Agr. Jour. India*, 15: 398-409. 1926.

<sup>15</sup> Barthel, C. and Bengtsson, N. *Meddel. Centralanst. Försöks. Jordbruk.*, 160, 172, 211, 269, 311. 1917-1926; *Fortschr. Landw.*, 1: H. 2. 1926.

manure in the form of organic compounds. According to Jensen,<sup>16</sup> 25 to 40 per cent of the nitrogen in the fresh, urine-free faeces of cattle and horses will be changed to nitrate in six months, in a moist soil. Other investigators<sup>17</sup> have also found that more nitrogen is changed to nitrate than would correspond to the ammonia in the manure.

In the process of composting of manure, the microorganisms bringing about the decomposition of the cellulose and pentosans consume a large part of the ammoniacal nitrogen for the building up of their cell substance; however, the microbes will also attack the organic nitrogenous complexes of the manure, liberating a part of the nitrogen in an available state. These two reactions take place under different conditions; at first the formation of microbial substance takes place at the expense of the inorganic nitrogen; this is followed later by the decomposition of the proteins and the liberation of some of the nitrogen as ammonia.<sup>18</sup>

TABLE 80  
*Nitrogen balance in the nutrition of sheep*

	grams
Nitrogen in oats (2.67 per cent N).....	79.6
Nitrogen in alfalfa hay (2.06 per cent N).....	91.5
Nitrogen in total feed.....	171.1
Nitrogen secreted in faeces (2.27 per cent N).....	65.4
Nitrogen secreted in urine.....	98.0
Nitrogen in total manure.....	163.4
Loss of nitrogen.....	7.7

The fact that a large part of the nitrogen may be stored away in the bodies of the microorganisms has been recognized.<sup>19</sup> According to Egorov, one-half to two-thirds of the nitrogen in the solid excreta of the manure is in the form of microbial cell substance. The wider the ratio of the energy material to the nitrogen, the less of the nitrogen will be rapidly nitrified.

The transformation of the nitrogen in the urine is so rapid that it can nearly all be changed, within a period of forty-eight hours, into ammo-

<sup>16</sup> Jensen, H. L. Jour. Agr. Sci., 19: 71-82. 1929; Sebelien, J. Nordisk. Jordbrugsförs, 3: 46-462. 1921.

<sup>17</sup> Glathe, H. Landw. Vers. Sta., 107: 65-129. 1927; Scheibe, K. Ibid, 108: 61-114. 1929.

<sup>18</sup> Heinze, B. Centrbl. Bakt. II, 25: 503-504. 1910.

<sup>19</sup> Wollny, E. Jour. Landw., 34: 213-320. 1886; Ramann, 1911 (p. XVI).

nium compounds. Since the nitrogen present in the urine makes up as much as a half of the total nitrogen in the manure and since the decomposition of the celluloses may be delayed for a certain time, in the composting of manure, this form of nitrogen represents an important source of loss, through direct volatilization.<sup>19a</sup> If the decomposition of the cellulose would set in quick enough, and if the ammonia nitrogen were immediately assimilated by the microorganisms bringing about the cellulose decomposition, the nitrogen would be transformed into organic complexes. The alkaline reaction, due to the formation of ammonium carbonate, may have something to do with the delay in the decomposition of the celluloses. Even when the decomposition of these carbohydrates sets in, it requires considerable time before all the available nitrogen in the urine is used up.

Another source of loss of nitrogen from the manure heap is found in the evolution of gaseous nitrogen, either through denitrification or through the chemical interaction of amino acids with nitrous acid, but this loss is comparatively small. Niklewski<sup>20</sup> found that the greatest loss of nitrogen from stable manure is a result of the action of the nitrifying bacteria. Only about 3 per cent of the nitrogen was lost, largely as ammonia, when manure was kept for 255 days free from nitrifying bacteria. In the presence of a large amount of urine, 5 to 12 per cent of the nitrogen was lost. Manure inoculated with nitrifying bacteria lost about 20 to 24 per cent of its nitrogen. This is due chiefly to the fact that the nitrates are reduced rapidly as soon as formed and elementary nitrogen is lost into the atmosphere.

According to Russell and Richards,<sup>21</sup> there is a distinct difference in the transformation of nitrogen compounds in the manure when stored under anaerobic or aerobic conditions (table 81). There was practically no loss of nitrogen under anaerobic conditions; the proteins were decomposed with the formation of ammonia, particularly at the higher temperature. Under aerobic conditions, there was a great loss of nitrogen, the amides having practically disappeared, while most of the ammonia was transformed into nitrites or nitrates. In general, under anaerobic conditions, the loss of dry matter and nitrogen is at a minimum; the gases formed consist of  $\text{CO}_2$ ,  $\text{CH}_4$ ,  $\text{H}_2$  and  $\text{NH}_3$ . Under aerobic conditions, there is a much greater loss of dry matter with a

<sup>19a</sup> Ehrenberg, P. Die Bewegung des Ammoniakstickstoffs in der Natur. P. Parey. Berlin. 1907.

<sup>20</sup> Niklewski, B. Centrbl. Bakt. II, 26: 388-442. 1910; 75: 206-213. 1928.

<sup>21</sup> Russell, E. J. and Richards, E. R. Jour. Agr. Res., 8: 495-563. 1917.

much greater decomposition of the nitrogenous compounds. Hydrogen and methane are not found in the gases. There is no ammonia accumulation, but nitrates are formed in the outer layers; with incomplete aeration, gaseous nitrogen is liberated.

Various groups of bacteria and fungi are active in the process of formation of ammonia in manure, but not with equal rapidity. Conn and Collison,<sup>22</sup> found that the strong proteolytic and gelatin liquefying bacteria like *Bac. cereus* and *Bact. fluorescens*, are not able to give off ammonia in quantities comparable to those given off from unsterilized manure. Conn has demonstrated that a minute, gram-negative, non-motile, non-spore forming rod, *Bact. parvulum* was able to give off from

TABLE 81

*Transformation of nitrogen in the composting of manure under aerobic and anaerobic conditions*<sup>21</sup>

	NH <sub>3</sub> -N	NO <sub>3</sub> -N	NO <sub>2</sub> -N	AMIDE-N	OTHER N COM- POUNDS (PROTEINS)	TOTAL
Anaerobic conditions						
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Start.....	28	..	..	8	64	100
6°C for 50 days.....	29	..	..	9	57	95
26°C for 50 days.....	44	..	..	7	47	98
Aerobic conditions						
Start.....	33	..	..	8	59	100
15°C.....	11	11	8	..	42	72
26°C.....	5	2	7	..	55	69

manure, in pure culture in the laboratory, amounts of ammonia, equal to or greater than those obtained from unsterilized manure. The decomposition of manure in the heap may not necessarily be carried on by the same organisms as in the soil. *Bact. fluorescens*, which was found to be relatively less active in the manure heap, was very active in the formation of ammonia from manure in soil.<sup>23</sup>

The Rothamsted experiments indicate<sup>24</sup> that during forty-seven years of application of manure to soil, 14 per cent of the nitrogen was accumu-

<sup>22</sup> Conn, H. J. and Collison, R. C. N. Y. Agr. Exp. Sta. Bul., 494. 1922.

<sup>23</sup> Conn, H. J. and Bright, J. W. N. Y. Agr. Exp. Sta. Tech. Bul., 67. 1919.

<sup>24</sup> Russell, E. J. 1927 (p. XVII); Lipman, J. G. and Blair, A. W. Soil Sci., 9: 371-392. 1920.

lated as humus, 25 per cent had been utilized by the plants and 60 per cent was lost, probably in the gaseous state, since there was very little drainage from the soil.

*Conservation of nitrogen in manure.* The problem of conservation of manure in the compost reduces itself to preventing the losses of ammonia. Liquid manure is alkaline in reaction, and since ammonium carbonate volatilizes rapidly, all the ammonia may be lost by volatilization. It is essential either to remove the  $\text{CO}_2$  or to substitute a strong non-volatile acid for the weak acid, so as to render the ammonia non-volatile. This can be accomplished by the addition of mineral acids ( $\text{HCl}$ ,  $\text{H}_2\text{SO}_4$ ) or acid reacting and buffering substances, such as superphosphate; in some cases sulfur has been recommended. The addition of a soluble salt, the cation of which forms a difficultly soluble carbonate ( $\text{CaCl}_2$ ,  $\text{Ca}(\text{NO}_3)_2$ ) and the anion of which combines with ammonia and keeps it in solution has also been recommended.<sup>25</sup> By hastening the process of decomposition of the carbohydrates in the manure, we hasten the conversion of the ammoniacal and other water soluble nitrogen into insoluble forms through the activities of the microorganisms. Possibly the liberation and transformation of the lignin are of importance in this connection, since lignin is known to conserve the ammonia in the urine.<sup>26</sup>

To prevent any loss through denitrification or the reduction of nitrates to atmospheric nitrogen, it is sufficient to stop the process of nitrification. This can be done either by preventing the inoculation of the manure with nitrifying bacteria, by making conditions anaerobic, or by adding acid reacting substances or disinfectants to stop the development of these bacteria.

The so-called "Edelmist" or "hot fermented manure" process, which consists in hastening the decomposition of the carbohydrates, until a temperature of  $65^\circ$  has been attained, is also directed towards diminishing the losses of nitrogen from the manure. In view of the fact that in the process of heating of the manure, very few bacteria, largely spores, are left, it is believed that this process is largely chemical in nature and not biological. Even if mesophilic and thermophilic bacteria are active

<sup>25</sup> Jensen, S. Tidskr. Planteavl., 34: 117-147. 1928; Wiss. Arch. Landw., 3: 161-180. 1930.

<sup>26</sup> Honcamp, F. and Wieszmann, H. Ztschr. Pflanz. Düng. Bodenk. A, 17: 194-199. 1930. Further information on conservation of manure is given by Lemmermann, O. and Wieszmann, H. Landw. Jahrb., 52: 297-341. 1918; Ruschmann, 1931 (p. 619).



in the early stages of decomposition, the latter stage is believed to be brought about largely by enzymes.<sup>27</sup>

*Transformation of potassium in manure.* The potassium content of barnyard manure varies from 0.5 to 3.0 per cent of the dry matter, or 3 to 20 pounds per 1 ton of fresh manure. Most of this potassium is water soluble and becomes available long before the organic matter of the manure is completely decomposed. Nearly all the potassium is present in plants in the form of salts. During the digestion of the plants in the animal system the salts are liberated, largely in the liquid excreta. The content of potassium in manure depends on the feeds and litter used and on the type and age of the animals. While leaching hardly affects the nitrogen and phosphorus content of the manure, the potassium was found to be reduced from 1.603 per cent to 1.154 per cent by leaching. This solubility is the reason for the ready availability of the potassium in manure. The potassium concentration in plants is controlled by the supply of available potassium in the soil; the dry matter produced by plant growth is, therefore, no accurate indication of the amount of available potassium in the soil. The addition of manure to soil usually leads to a reduction in the total and available potassium content of the soil, probably due to the fact that the decomposition of the manure makes some of the potassium of the soil available to the growing plants.<sup>28</sup> The transformation of phosphorus by micro-organisms is discussed in detail elsewhere (p. 571).

*Microbiological population of manure.* Stable manure harbors organisms partly derived from the intestinal contents of the animal<sup>29</sup> and partly introduced into the digestive system with the feeds. After a certain period of decomposition, whether in the compost heap or in the soil, there is a tendency for certain intestinal organisms in the manure to die out and for other organisms, more adapted to the new surroundings, especially to the changes in aeration and temperature, to take their place.

The importance of bacteria in manure consists in beginning the process of decomposition. If their activities are not controlled and stopped in time, as in the "hot fermentation" process, the decomposition may

<sup>27</sup> Ruschmann, H. *Centrbl. Bakt.* II, 70: 214, 383. 1927; 72: 193. 1927; *Fortschr. Landw.*, 2: 46. 1927; *Landw. Jahrb.*, 67: 211. 1928; Ehrenberg, P. *Ztschr. Pflanzen. Düng. Bodenk. B*, 9: 49-67. 1930.

<sup>28</sup> Bartholomew, R. P. *Jour. Amer. Soc. Agr.*, 20: 55-81. 1928.

<sup>29</sup> Choukevitch, J. *Ann. Inst. Past.*, 25: 247-276. 1911; Hopfe, A. *Centrbl. Bakt. I or.*, 83: 376. 1919; Strelkov, A. *Arch. Protistenk.*, 68: 503-555. 1929.

go too far; the losses become larger and larger, the manure becomes overripe and its action is diminished. The better results obtained with this manure proves that the mere addition of bacteria in the manure to normal soil is unnecessary. However, in the case of poor sands, newly cultivated peats, or in heavy soils, the bacteria of a 6 to 8 week old manure, not "fermented hot," may contribute to the bacterial activities in the soil. Generally, however, manure stimulates soil fertility, not by increasing bacterial numbers but by creating physical and chemical conditions favorable for the microbial world. Even the bacteria-poor, but well decomposed organic residues of the "hot manure," favor an increase in soil temperature and in the moisture-holding capacity of the soil, thus making conditions favorable for the development of microbial life. It has been suggested<sup>30</sup> that the favorable effect of a manure compost added to green manure, when the latter is turned under, is due to the bacterial content of the compost.

According to Löhnis<sup>30</sup> the initial rôle of bacterial activities in stable manure is highly favorable, while a more continued bacterial development is injurious, not only because of the nitrogen losses, but because of the fact that plant nutrients are synthesized into bacterial cells, which are later decomposed in soil only very slowly. In the case of hot manure, the heat and lack of oxygen bring about a rapid destruction of the bacteria. Within a few weeks, at 55–65°C., and optimum moisture, the bacterial cells decompose rapidly; as a result of chemical interaction of the ammonia and amino acids with carbohydrates, humus compounds are formed, of a neutral reaction, which are rapidly decomposed in soil to nitrate and CO<sub>2</sub>. However, Gerlach and Seidel obtained totally different results on comparing the rapidity of decomposition of hot and cold fermented manures.

A detailed discussion of the influence of stable manure on the microbiological activities in soil is given elsewhere (p. 744).

*Manure inhabiting fungi.* The manure inhabiting or coprophilous fungi do not form one specific group of organisms, but are widely distributed among the various families of fungi. Many of these organisms live on animal excreta as upon a specific substrate and occur only seldom on other substrates; in the case of some of the fungi, the passage through the intestinal canal of an animal is necessary to induce germination. Saccardo records 757 species of coprophilous fungi belonging to 187 genera; some occur only on manure, while others are able to live also on

<sup>30</sup> Löhnis, F. *Illustr. landw. Ztg.* 1928, p. 132. *Fortschr. Landw.*, **4**: 65. 1929; Gerlach and Seidel. *Ztschr. Pflanzen. Düng. Bodenk.*, **8B**: 15. 1929.

decomposing organic matter in soil. The manure of the herbivorous animals is richest in fungi. When fresh manure is placed under a bell-jar, the sequence of fungus developments is as follows: the *Phycomyces* (*Pilaira* and *Pilobolus*, then *Mucor*) develop first, accompanied by their parasites *Chaetocladium*, *Thamnidium*, *Piptocephalis*, and *Syncephalis*; next appear various *Hyphomycetes*, followed by the ascigenous fungi.<sup>31</sup>

A number of fungi are capable of adapting themselves to the high temperatures which the manure may attain. Species of *Coprinus*, *Asp. fumigatus* have their optimum at 40°C. and can survive a long time at 55°C. Some of the actinomyces can even live at 57°–60°C.<sup>32</sup>

The available information concerning the manure inhabiting fungi is distributed through an extensive mycological literature. These fungi can be classified as follows:

MYXOMYCETES, represented by the genera *Copromyza*, *Dictyostelium*, *Cornovia*, *Chondrioderma*, etc.

MUCORACEAE, including a number of species of *Mucor* (*M. racemosus*, *M. plumbeus*, *M. circinelloides*, etc.), *Circinella*, *Phycomyces*, *Rhizopus* and *Absidia*; THAMNIDIACEAE, including species of *Thamnidium*, *Chaetostylum*, *Helicostylum*. PILOBOLACEAE, including species of *Pilaira* and *Pilobolus*; CHAETOCLADIACEAE and CEPHALIDACEAE.

ENTOMOPHTHORACEAE (*Basidiobolus*); ASCODESMIDACEAE; PEZIZACEAE (*Humaria*, *Pustularia*, *Lachnea*); AXOBOLACEAE (*Thelebolus*, *Rhyparobius*, *Lasibolus*, *Ascophanus*, *Saccobolus*, *Boudiera*, *Ascobolus*); SACCHAROMYCETACEAE (*Saccharomyces*), GYMNOASCACEAE (*Arachniotus*, *Gymnoascus*, *Myxotrichum*, *Amuroascus*), ASPERGILLACEAE; ONYGENACEAE; PERISPORIACEAE, HYPOCREACEAE (*Sphaeroderma*, *Melanospora*) and CHAETOMIACEAE.

SORDARIACEAE (*Hypocopra*, *Sordaria*, *Philocopra*, *Coprolepa*, *Delitschia*, *Sporormia*); SPHAERIACEAE (*Rosellinia*), XYLARIACEAE (*Poronia*, *Xylaria*) and AURICULARIACEAE (*Platyglœa*).

AGARICACEAE (*Coprinus*, *Bolbitius*, *Coprinarius*, *Chalymotta*, *Anellaria*, *Psilocybe*, *Psalliota*, *Derminus*).

MUCEDINACEAE (*Oedocephalum*, *Cephalosporium*, *Eidamia*, *Coemansiella*, *Aspergillus*, *Penicillium*, *Martensella*, *Chaetoconidium*,

<sup>31</sup> Massee, G. and Salmon, E. S. Ann. Bot., 15: 313–357. 1901; 16: 57–93. 1902.

<sup>32</sup> Perrier, A. Compt. Rend. Acad. Sci., 188: 1426–1429. 1929.

*Acremonium*, *Coemansia*, *Acrostalagmus*, *Clonostachys*, *Spicaria*, *Trichothecium*, *Arthrobotrys*, *Fusoma*); DEMATIACEAE (*Echinobotryum*, *Stachobotrys*, *Botryotrichum*, *Stemphylium*, *Alternaria*); STILBACEAE (*Coremium*, *Isaria*, *Graphium*, *Sporocybe*, *Stysanus*); TUBERCULARIACEAE (*Fusarium*) and NECTRIOIDACEAE (*Sphaeronaemella*).

In summarizing the occurrence of coprophilic fungi in Silesia, Schmidt<sup>33</sup> reported 4 species belonging to the Myxomycetes, 42 to the Phycomycetes, 100 to the Ascomycetes, 21 to the Basidiomycetes and 35 to the Fungi Imperfecti.

Most of the fungi found in Germany were also found in other parts of Europe, as well as in America and in Africa. The most commonly distributed coprophilic fungi are *Ascobolus stercorarius*, *A. immersus*, *Saccobolus kerverni*, *S. depauperatus*, *Lasiobolus equinus*, *Ascophanus carneus*, *Thelebolus stercoreus*, *Rhyparobius crustaceus*, *Hypocopra fimicola*, *H. discospora*, *Sordaria fimiseda*, *S. curvula*, *Sporormia minima* and *Sp. intermedia*.

Schmidt divided the manure inhabiting fungi into 3 groups:

1. Those that are found only in manure; their spores are swallowed by the animals with the feed; they pass unchanged through the digestive tract and are favorably influenced by the body heat and digestive fluids of the animal towards germination. They usually do not germinate at ordinary temperatures, and not even at higher temperatures in artificial solutions. The combined action of heat and chemicals favors germination. Multiplication by spores is impossible without the physiological action of the digestive processes. Among these fungi are *Lachnea stercorea*, *Ascobolus perplexans*, *A. stercorarius*, *A. immersus*, *Saccobolus depauperatus*, *Myxotrichum uncinatum*, etc.

2. The representatives of this group as well occur in nature only in manure, although some are able to grow also on other substrates. They can be cultivated both on manure and on other media, mostly at ordinary temperatures. The passing through the digestive tract of an animal is not essential for most of them. They are distributed by mammals, insects and wind. Here belong *Rhyparobius albidus*, *Rh. pachyascus*, *Ascophanus carneus*, *Thelebolus stercoreus*, species of *Sordaria*, *Pilaira anomala*, *Pilobolus kleinii*, *P. crystallinus*, *P. roridus*, *P. longipes*, and various species of *Mortierella*, *Piptocephalis* and *Syncephalis*, etc.

3. Organisms belonging to this group are found in manure and on other substrates. They can be cultivated at room temperature on a number of media. They are widely distributed by wind, insects and mammals. Here belong species of *Mucor*, *Circinella*, *Thamnidium*, *Helicostylum*, *Absidia*, *Mortierella*, *Chaetocladium*, *Microascus*, *Chaetomium* and *Pilobolus aedipus*, as well as manure inhabiting *Agariceae* and *Fungi imperfecti*, and forms of *Arachniotus* and *Gymnoascus*.

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<sup>33</sup> Schmidt, A. Die Verbreitung der coprophilen Pilze Schlesiens. Inaug. Diss. Breslau. 1912.

*Manure inhabiting bacteria.* Numerous contributions have been made to the subject of the bacterial population of stable manures and of the changes in the numbers and kinds of bacteria, under different processes of composting and at various stages of decomposition of the manure.<sup>34</sup> The temperature and aeration of the compost are among the most important factors controlling the abundance and types of bacteria. This can well be illustrated by citing the results obtained with the so-called "Edelmist" process. This term has been used to designate a special process of treating manure modified by Krantz, whereby the manure is at first piled up loosely in shallow layers and allowed to undergo active decomposition, due to proper aeration; the temperature begins to rise rapidly within a few days. When it reaches 55°–65°C, the heap is thoroughly compacted by tramping, and is covered with a loose layer of fresh manure which is allowed to decompose further, thus gradually building up the heap (from 6–9 to 18–20 feet). At that time the heap is well tramped down and is allowed to remain undisturbed for 3–4 months. During the heating of the manure, the microorganisms active at ordinary temperatures are gradually destroyed and are replaced by thermophilic organisms which are believed to require an abundance of air for their activities. When the compost is compacted, the air is excluded and the thermophilic bacteria become inactive, thus leading to a gradual drop in temperature. The straw in the manure thus prepared is found to be well broken down. The high temperature also favors the destruction of weed seeds and pathogenic bacteria.<sup>35</sup>

Goeters<sup>36</sup> prepared a compost of fresh manure, fresh urea and straw, in a ratio of 42:7:9 and containing 26.3 per cent dry matter and 0.39 per cent total nitrogen. By the use of meat peptone agar for determining the total numbers of bacteria, 940,000,000 cells were found in 1 gram of fresh compost. In the same quantity of compost, he demonstrated 100,000,000 urea-decomposing, 10,000,000 anaerobic cellulose-decomposing, and 1,000,000 aerobic cellulose decomposing organisms. When the manure was kept at 20°C., the numbers diminished from 940 millions per gram to 760 millions in six hours, and to 230 millions in 3 days. The

<sup>34</sup> See p. 26. A detailed review of the earlier literature is given by Löhnis, 1910 (p. XIV); Scheffler, W. Landw. Jahrb., 42: 429–547. 1912; Ruschmann, G. Centrbl. Bakt. II, 70: 214–260, 383–411; 72: 193–236. 1927; 73: 179–206; 75: 182–205, 405–426. 1928; 77: 216–239. 1929; Weigert, J. Ztschr. Pflanzenernähr. Düng. 5B: 145. 1926, Mangold, 1929 (p. 619).

<sup>35</sup> Cunningham, A. Scott. Jour. Agr., 10: 434–439. 1927.

<sup>36</sup> Goeters, W. Landw. Vers. Sta., 108: 1–60. 1929; Glathe. Ibid., 107: 65–129. 1927.

numbers then increased gradually, reaching 600 millions in 7 days, 810 millions in 14 days, and 3,300 millions in 28 days. A half of these bacteria were in the spore stage. The urea-decomposing organisms showed similar changes, while the aerobic cellulose-decomposing bacteria increased during the first few days, then gradually diminished.

At 40°C, there was an increase in bacterial numbers during the first 24 hours (214 per cent), then a decrease in 3 days (50 per cent); this was followed by another increase reaching a maximum in 14 days (800 per cent); only 6.8 per cent of the cells were in a spore stage. The urea bacteria changed in an almost parallel manner. The aerobic and anaerobic cellulose decomposing bacteria at first increased, then diminished, and practically disappeared within 4 weeks. When the manure was kept at 60°C. (determinations made at 38°C.), all bacteria diminished gradually, namely to 75 per cent in 6 hours, to 69 per cent in 24 hours, to 20 per cent in 3 days, to 5 per cent in 7 days and to 2 per cent in 4 weeks. The urea and cellulose decomposing bacteria also diminished in numbers very rapidly. When the manure was kept at 60°C., and the numbers of bacteria determined at 55°C., a thermotolerant and a thermophilic flora of bacteria was found to take the upper hand gradually. Many of the urea and anaerobic cellulose-decomposing organisms are known to have thermotolerant properties.

The total number of bacteria in manure rapidly decreases at very high (60–80°C.) or at very low (0°C.) temperatures. The greatest multiplication takes place at 20–40°C. Higher temperatures kill most of the vegetative cells, leaving largely the spores, with the result that the less numerous obligate thermophilic organisms gradually increase at 60°C. The urea bacteria diminish somewhat on the heating of the manure, but these forms are among the most heat resisting organisms and seem to play a very important rôle in the ripening of the manure. The anaerobic cellulose-decomposing bacteria are quite abundant in the inner parts of the manure heap and are less resistant to high temperatures than the other bacteria, while the aerobic cellulose decomposing organisms develop only at temperatures less than 38–40°; above that temperature they are rapidly destroyed. Manure composted under ordinary conditions of the stable undergoes a rapid decomposition and loss of nutrients due to the high bacterial content.

Among the various heterotrophic bacteria commonly found in the manure, Ruschmann reported the presence of *Bac. vulgatus*, *Bac. subtilis*, *Bac. mesentericus*, *Bac. ellenbachensis*, *Bac. graveolens*, *Bac. petasites*, *Bact. fluorescens*, *Bact. putidum*, *Bact. enteritidis*, *Bact. coli*, *Bact. paracoli*, *Bact. flavum*, *Bact. vulgare*,

*Micr. luteus*, *Micr. candidans*, *Micr. sulfureus*, *Micr. aurantiacus*, *Micr. pyogenes albus*, *Sarcina flava*, *Strept. pyogenes*.

Among the specific organisms we find the nitrifying and certain denitrifying bacteria. Various thermophilic bacteria are found in the intestinal tract of animals;<sup>37</sup> this is true especially of the cellulose-decomposing organisms in herbivora, especially in man,<sup>38</sup> herbivorous animals and insects.<sup>39</sup> The myxobacteria are also found constantly growing in the manure heap (p. 154). Pathogenic bacteria may be found frequently in stable manure; here belong the *B. tuberculosis* and various intestinal parasites.

*Coprophilic protozoa.* The presence of numerous protozoa in the intestinal tract of animals has been definitely established. Some of these are parasitic while most are saprophytic. Minchin<sup>40</sup> originally used the term *coprozoic* to designate those protozoa which are carried through the alimentary canal in the form of cysts; they were believed to become active only in the moist manure. More recently, the term has been applied to all organisms capable of living in faeces. Alexeiev<sup>41</sup> suggested to designate those coprozoic protozoa, or the protista-coprocolae, which are voided with the faeces, as *endogenous* and those that contaminate it from outside as *exogenous*. Some protozoa pass through the digestive system in the form of cysts, in an uninjured condition, while others actually lead a vegetative existence in the animal body. It has even been suggested that some protozoa live symbiotically with certain animals, as in the case of the termites, which are believed<sup>42</sup> to be capable of digesting cellulose and wood only through the agency of protozoa living in their digestive tract. Alexeiev believed that protozoa may play a highly important part in the manure by assimilating the nitrogen compounds and fixing them in their bodies, thus preventing volatilization of ammonia.

Among the protozoa which are capable of developing in manure and in urine are found not only saprophytic forms but also certain parasites, such as *Trichomastix* and *Trichomonas*, which are capable of living in the excreta and even multiply under these conditions. The coprophilic protozoa comprise largely various flagellates (*Bodo edax*, *Monas*), certain amoebae (*A. limax*), and *Col-*

<sup>37</sup> Black, L. A. and Tanner, F. W. Centrbl. Bakt. II, 75: 360-375. 1928.

<sup>38</sup> Khourvine, 1923 (p. 188); Kohmoto, T. and Sakaguchi, S. Jour. Biochem. Japan., 6: 61-76. 1926.

<sup>39</sup> Werner, 1926 (p. 189).

<sup>40</sup> Woodstock, H. M. Phil. Trans. Roy. Soc., 207B: 375. 1916; Dobell, C. and O'Connor, F. W. The intestinal protozoa of man. London. 1921.

<sup>41</sup> Alexeiev, A. G. Jour. Microbiol. (Russian) 4: 97-134. 1917.

<sup>42</sup> Cleveland, L. R. Amer. J. Hyg., 4: 444-461. 1923; Quart. Rev. Biol., 1: 51-60. 1926; Biol. Bul., 54: 231-237. 1928.

*pidium colpoda*. The liquid portion of the manure is considerably richer both in total numbers and in species; here belong *Polytoma uella*, *Cryptochilum nigricans* and *Tetramitus rostratus*. They nearly all feed upon bacteria. The infusoria may feed upon smaller protozoa, so that organisms like *Colpidium* may not destroy bacteria at all.

Hoare<sup>43</sup> described three ciliates, *Lembus pusillus*, *Cyclidium glaucoma* and *Uronema nigricans*, which feed exclusively on bacteria and which can be grown successfully upon human and animal faeces. They are coprozoic forms that had contaminated the material examined.

*Preparation of artificial manures.* The principle underlying the preparation of artificial manure from plant residues consists in creating conditions favorable for the decomposition of a part of the cellulose and hemicelluloses in these residues by microorganisms and in the conversion by the cells of inorganic nitrogen compounds into complex organic substances. To a plant product, such as cereal straw, high in carbohydrates but low in nitrogen, are added simple, usually inorganic, compounds of nitrogen (ammonium salts, nitrates, cyanamide, urea), some available phosphate and potassium salts, and calcium carbonate. The compost is brought to an optimum moisture content by the addition of water, so as to make conditions favorable for the activities of the fungi and bacteria. Decomposition of certain of the constituents of the straw sets in rapidly, with an abundant development of various microorganisms. These consume the plant constituents, largely the cellulose and hemicelluloses as sources of energy and build up considerable cell substance, utilizing thereby the nitrogen and other minerals added to the compost. A considerable reduction of the bulk of the straw takes place with the generation of a large amount of heat, as shown in fig. 56. Some of the constituents of the straw decompose more rapidly than others, with the result that the compost, or the so-called artificial manure, is distinctly different in composition than the original plant material.

The decomposition of mature straw and other plant residues in composts leading to the formation of so-called artificial manure involves a knowledge of the composition of the plant materials, of the mechanism of the decomposition processes which are brought about by microorganisms, and of the metabolism of these organisms.

Straw and other farm residues which are commonly used for the purpose of composting consist predominantly (60 per cent or more) of cellulose and hemicelluloses, which undergo rapid decomposition in the presence of sufficient nitrogen and other minerals; of lignin (15 to 20 per

<sup>43</sup> Hoare, C. A. *Parasitol.*, 19: 154-222. 1927.



cent) which is more resistant to decomposition and which gradually accumulates, either in the original form or as various transformation products; of water soluble substances (5 to 12 per cent) which decompose very rapidly; of proteins which are usually present in very small amounts (1.2 to 3.0 per cent) but which gradually increase in concentration with the advance of decomposition; and of the mineral portion or ash.

The carbohydrates of the straw cannot serve as direct sources of energy for the nitrogen-fixing bacteria, and their decomposition depends

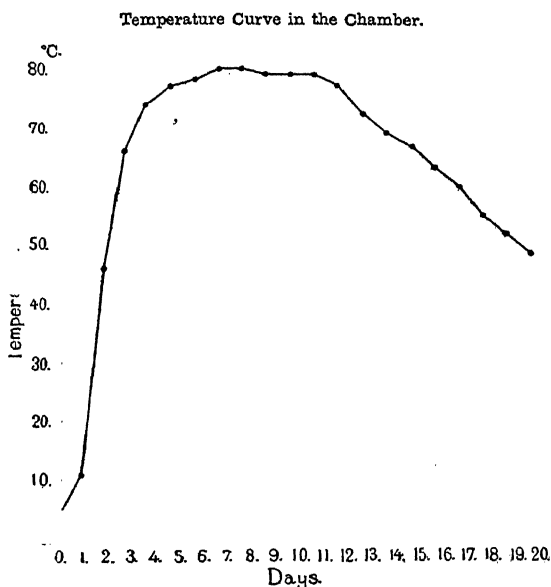


FIG. 56. Temperature changes in the composting of artificial manure (from Itano).

entirely upon the action of various fungi and aerobic bacteria, which need a considerable amount of nitrogen for the synthesis of their cell substance. The latter may be quite considerable, and is frequently equivalent to as much as a third of the total organic matter decomposed. The microorganisms require the nitrogen and phosphorus for the building up of the proteins and the nucleins in the microbial cells. Since there is a direct relation between the cellulose decomposed and the cell substance synthesized, one may suggest also a relation between the amount of carbohydrates decomposed and of the nitrogen required by the organisms, which is about 40-50:1. This explains the increase in

the protein content of the compost, accompanying the gradual decrease of the cellulose and hemicelluloses, as shown in figs. 51 and 53, as well as in fig. 57.

The advantage of adding this compost or "artificial manure" to the soil instead of the mixture of straw and the mineral salts is manifold. As shown previously (p. 613), the injurious effect of addition of straw to soil upon the following crop consists largely in the competition between the growing plants and the microorganisms decomposing the carbohydrates of the straw, for the available nitrogen; however, after the straw has been composted for some time this effect will not be observed. Secondly, the nitrogen is present in the compost not in an inorganic form but as organic nitrogen, hence it will not be subject to leaching or to other losses, to which that part of the inorganic nitrogen added to the soil which is not rapidly assimilated is subject. The "artificial manure"

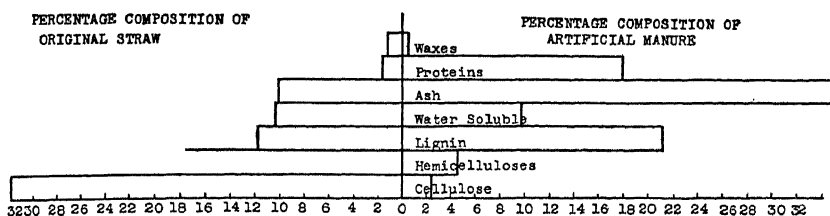


FIG. 57. Relative composition of initial plant material and of artificial manure after 273 days of decomposition at 37°C. (from Waksman and Gerretsen).

is similar in every respect to composted stable manure, both chemically (table 82)<sup>44</sup> and in its action upon plant growth. "Artificial manure" may also be prepared so as to take the place of stable manure for the growth of mushrooms,<sup>45</sup> although much remains to be learned in this connection.

The rapidity of decomposition of the plant residues in the compost of "artificial manure" depends upon 1. the nature of the plant material, 2. the amount and nature of available nitrogen, 3. the presence of other minerals, especially phosphorus and calcium carbonate, 4. the amount of moisture added, and 5. the temperature of decomposition.

The composting of straw with inorganic salts has been known since

<sup>44</sup> Waksman, S. A., Tenney, F. G. and Diehm, R. A. Jour. Amer. Soc. Agron., 21: 533-546. 1929.

<sup>45</sup> Lambert, E. B. Science, Aug. 2, 1929.

1872.<sup>46</sup> In 1919, Lemmermann<sup>47</sup> stated that the reasons for the need of additional available inorganic nitrogen to bring about the decomposition of straw is due to the fact that the latter contains an insufficient amount of nitrogen, most of which is not fully available, for the activities of the microorganisms; these must obtain this nitrogen from an outside source, either from the soil, thus bringing about a competition with higher plants, or from added nitrogen. Rahn and Lemmermann<sup>48</sup> have found that when cereal straw was composted without the addition of inorganic nitrogen, there was a loss, in four weeks, of 9.0 per cent of the dry material; when composted with 0.15 per cent of nitrogen, the loss was 14.0–16.4 per cent, and, when composted with 0.30 per cent nitrogen, the loss

TABLE 82

*Relative composition of "artificial manure" from straw and of horse manure composted under exactly the same conditions and for the same period of time*

On the basis of dry matter

PLANT CONSTITUENTS	ARTIFICIAL MANURE"	COMPOSTED HORSE MANURE
	<i>per cent</i>	<i>per cent</i>
Ether-soluble.....	0.41	0.95
Soluble in cold and hot water.....	8.73	5.71
Hemicelluloses.....	14.77	12.67
Cellulose.....	13.75	5.97
Lignin.....	24.39	28.43
Protein.....	14.56	16.38
Ash.....	19.44	19.32
Total.....	96.05	89.43

increased to 16.9–20.5 per cent. The limited reduction in the bulk of the straw with no additional nutrients is due both to the decomposition of the water-soluble carbohydrates (this process being independent of the supply of available nitrogen in the soil, since it can be carried out by the nitrogen-fixing bacteria) and to the presence of small amounts of protein

<sup>46</sup> Bergstrand. Preusz. Ann. Landw., 1872, p. 231 (Ref. Löhnis, 1910 (p. XIV, p. 499); Hébert, 1892 (p. 621)).

<sup>47</sup> Lemmermann, O. Arb. deut. landw. Gesell. H. 297: 56–60. 1919; Centrbl. Bakt. II, 41: 608–625. 1914.

<sup>48</sup> Rahn, O. Ztschr. techn. Biol., 7: 172–186. 1919; Lemmermann, O. and Gerdum, E. Ztschr. Pflanz. Düng. Bodenk. B, 6: 481–485. 1927; Lemmermann, O., Jessen, W. and Engel, H. Ibid, 17: 321–355. 1930.

in the straw. The more considerable decomposition of the straw which took place in the presence of inorganic nitrogen compounds was no doubt due to the attack by the microorganisms of the more complex polysaccharides.

Hutchinson and Richards<sup>49</sup> added to every 100 parts of straw 0.7 parts of nitrogen; urea and ammonium carbonate were found to be the most appropriate sources of nitrogen for this purpose; cyanamide could also be employed, while ammonium sulfate proved efficient only in the presence of additional  $\text{CaCO}_3$ . The addition of 600 liters of water containing 4 kgm. of urea to 250 kgm. of straw, with the moisture content of the mixture adjusted to 70 per cent, gave a favorable decomposition in three months. The artificial manure thus prepared contained 20.5–26.3 per cent dry matter, 0.43–0.44 per cent nitrogen, 15.0–19.4

TABLE 83

*Nitrate formation from "artificial manure" as influenced by its nitrogen content*

	NITROGEN CONTENT OF COMPOST	NITRATE FORMED IN FOUR WEEKS
	<i>per cent</i>	
Soil alone.....		100.0
	1.48	47.1
	1.60	84.0
Soil + compost.....	1.80	136.9
	2.07	211.1
	2.32	258.2

per cent organic matter and a C/N ratio of 17:1 to 22:1. The analysis of ordinary stable manure, on a similar basis, gave 20.3–23.0 per cent dry matter, 0.46–0.55 per cent nitrogen, 15.0–17.0 per cent organic matter, and a C/N ratio of 15:1 to 16:1.

Numerous other studies tended to show that the addition 0.5 to 0.75 per cent of nitrogen, and an incubation period of about six months will result in the preparation of a good manure. A mixture of 60 pounds of ammonium sulfate, thirty pounds superphosphate, 25 pounds potassium chloride and 50 pounds of ground limestone, added to each ton of dry straw was found<sup>50</sup> to result, after composting for 4 to 6 months at an optimum moisture content, in a good manure.

<sup>49</sup> Hutchinson, H. B. and Richards, E. H. Jour. Ministry Agr., 28: 398–411. 1921.

<sup>50</sup> Collison, R. C. and Conn, H. J. New York Agr. Exp. Sta. Circ. 95. 1928; Albrecht, W. A. Mo. Agr. Exp. Sta. Bul. 258, 1927.

The "artificial manure" with a low nitrogen content, just as composted stable manure free from soluble nitrogen, does not offer a readily available nitrogen source. However, a compost prepared with a high nitrogen content, some of which is in a soluble form, is similar in its behavior in soil to stable manure, and its nitrogen will become just as readily available, as shown in table 83.<sup>51</sup>

When, instead of straw, molasses is used as a source of energy, for preparing an "artificial manure" a number of microbiological processes are brought about, different from those taking place in straw composts; those include possibly non-symbiotic fixation of nitrogen due to the high content of available sugar.<sup>52</sup>

<sup>51</sup> Flieg, O. Ztschr. Pflanzen. Dung. Bodenk. B, 9: 1-15. 1930.

<sup>52</sup> Moir, W. W. G. 48th Ann. Meet. Proc. Hawaiian Sugar Plant. Assoc. (1928); 135-165. 1929.

## CHAPTER XXVI

### CHEMICAL AND MICROBIOLOGICAL PROCESSES INVOLVED IN THE TRANSFORMATION OF ORGANIC MATTER IN PEAT BOGS AND PEAT SOILS

*Nature and formation of peat.* The term peat is applied to accumulations of organic matter on the surface of the earth, as a result of incomplete and partial decomposition of plants growing either below or above the surface of water. The anaerobic conditions, resulting from the saturation of the medium in which the plants are growing with water, prevent the development of fungi and aerobic bacteria, except at the very surface in contact with the air; the activities of the anaerobic bacterial flora predominating in the peat bog are not sufficient to bring about complete decomposition of the plant remains, with the result that these accumulate in a more or less modified form thus giving rise to peat.

The nature of the peat depends primarily upon the nature of the plants which gave rise to it, while the latter are controlled by the mineral composition and reaction of the waters in which they are growing. The chemical composition of peat depends upon the chemical composition of the plant associations from which it has originated, environmental conditions under which decomposition has taken place, and the microorganisms bringing about the decomposition processes.

The physical properties of peat are best expressed in a definition proposed by one of the outstanding students of the subject, Weber.<sup>1</sup> According to this definition, "peat is an organic mineral formed out of dead cellulose rich plants, by a special process of decomposition (ulmification) or peat formation; it becomes colored brown to black when exposed to air; it is soft in its natural moist condition and is very rich in water; its specific color depends on its content of "ulmin." Peat consists largely of the elements carbon, hydrogen, oxygen, and contains, in addition varying amounts of nitrogen, sulfur and ash. Various animal residues are admixed in varying amounts in peat, in the form of excreta and chitin. On drying, peat shrinks quite considerably, giving fragments hanging loosely together or hard clumps, breaking apart with

<sup>1</sup> Weber, C. A. Abhandl. Naturw. Ver. Bremen, 17: 465-484. 1903.

the formation of sharp edges or into fibrous or felt-like masses. The air dry substance swells, depending on the nature of the constituent plant residues, on the degree of decomposition and on the pressure to which it has been subjected during the continuous contact with water; it never gives, however, a crumbly soil-like mass even when completely softened. According to the degree of "ulmification" and the manner in which peat has been formed, the plant residues from which the peat has been formed can either still be recognized by the naked eye or by use of magnification."

The fundamental factors which influence the origin and nature of peat are predominantly functions of climate, and of soil and water relations. They influence the formation of a typical vegetation as well as the type of its decomposition. These factors determine the nature of the particular type of peat produced.<sup>2</sup> Peat will be formed on land which is poorly drained and where water may collect and stand permanently. Peat forming plants find these conditions favorable for their development, especially when the high humidity of the air prevents evaporation and the temperature is low. Under these conditions, the decomposition processes are very slow and the partly decomposed plant residues give rise to peat.

*Classification of peat.* Various systems of classification of peat have been proposed and used at various times. These classifications have been based either upon, 1. the nature of plant associations on the surface of the peat; 2. the fact whether the peat was formed below or above the surface of the water, from plants growing in situ (autochthonous) or brought in from outside (allochthonous); 3. the higher or lower content of plant nutrients available to the growing plants: lowmoor peats derive their water supply by drainage from surrounding hills or upper lands, which are rich in nutrients, while the highmoor peats derive their water supply only by precipitation, and are therefore poor in nutrients; 4. the physical and mechanical properties of the peat, whereby it is classed as fibrous, sedimentary, colloidal;<sup>3</sup> 5. recently the tendency has been to classify peat on the basis of the plants which gave origin to it.<sup>4</sup>

The four predominant types of peat can be summarized botanically as follows:

1. Lowmoor peat (torf, marsh, fen, reed-peat, sedge-peat, Niederungs- or

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<sup>2</sup> Birk, C. Arb. Lab. techn. Moorkerwert. K. Techn. Hochsch. Hannover, 1: H. 1. 1914.

<sup>3</sup> Schreiber, H. Moorkunde nach dem gegenwärtigen Stande des Wissens auf Grund 30-jähriger Erfahrung. P. Parey. Berlin. 1927.

<sup>4</sup> von Post, L. Sver. Geol. Unders. C, 19: No. 4. 1926.

Niedermoor, Flachmoor). The dominant and characteristic species of plants in this peat are sedges, reeds and certain trees and shrubs (species of *Phragmites*, *Cladium*, *Scirpus*, *Juncus*, *Equisetum*, *Hypnum*, *Alnus glutinosa*, *Betula alba*, etc.). Sphagnum plants are absent or rare. This type of peat occurs chiefly in river valleys and is fed by ground waters which are rich in mineral salts. Frequently this type is subdivided, largely upon the basis of the predominant type of plants, such as *Carex* peat, *Cladium* peat, *Hypnum* peat, *Phragmites* peat, etc. This peat is usually formed in places where the waters, containing calcium and rich in nutrients, are slowly draining into lower regions or inclined planes. The waters are of telluric origin.

2. Highmoor peat (moor-peat, moor, heath, sphagnum peat in strict sense, Hochmoor). The predominant vegetation consists of the following: *Pinus silvestris*, *Cassandra calyculata*, *Ledum palustra*, *Andromeda polifolia*, *Eriophorum vaginatum*, *Rhynchospora alba*, *Scheuchzeria palustris*, *Sphagnum medium*, *S. recurvum*, *S. balticum*, *Calluna vulgaris*, etc. This type of peat is formed in waters poor in mineral nutrients and containing little calcium; the waters originate either from atmospheric precipitation or from mineral poor soils. The highmoor peat may be formed either upon a lowmoor peat, upon a forest peat or directly upon sand, clay or rock. This type is usually formed in cold and moderate climate with high rainfall. Different species of Sphagnum prefer different moisture conditions, the *S. cuspidatum* growing in regions of higher moisture, and the *S. medium* and *S. acutifolium* in regions of lower moisture.

3. Forest peat (forest swamp, transition peat). This peat is formed by the following plants: *Betula*, *Quercus*, *Alnus*, *Pinus*, *Picea*, with an admixture of *Calluna*, *Oxycoccus*, *Salix*, *Andromeda*, *Carex lasiocarpa*, *Calamagrostis lanceolata*. Various species of sphagnum (*S. recurvum* and *S. subicolor*) form a continuous carpet. This type of peat occurs at the end of sphagnum bogs and is fed partly by ground waters and partly by precipitation; these waters are less rich in salts than the lowmoor peats; they usually develop along the upper courses of rivers.

4. Sedimentary or lake peat (Gyttja). In addition to the above three general types of peat, there are various combinations of peat as well as certain minor types. The most important among these is the lake of mud peat, which is formed largely by algae and other aquatic plants and animals (insect shells), with an admixture of spores, pollen and particles of clay and sand. It is usually found in the lowest layers of the peat bog.

It is of interest to mention here also the heath-peatland (*Vaccinium-Calluna* association) which is called peat incorrectly and is more a "raw humus" formation similar to "alpine humus."

This marked difference in the botanical composition of the different peats is accompanied by important differences in the chemical composition of the organic complexes, as well as in the reaction and abundance of mineral elements.

The stratification of peat deposits is directly related to the changes in the wet and dry periods which accompany changes in climatic conditions.



A vertical cut of a peat profile may give the following strata or layers,<sup>5</sup> from the surface to the bottom:

Highmoor stage	{ Heath-peatland Sphagnum peat Eriophorum-sphagnum peat
Transition stage	{ Eriophorum peat Scheuchzeria peat Alder, eventually birchwood peat
Lowmoor peat	{ Carex peat, finally Hypnum peat Phragmites peat
Sedimentary peat	{ Lake peat or Lebertorf (algae and other lower plants and animals) Mineral mud layer (diatoms, etc.)

The climatic and physiographic conditions which favor the accumulation of plant residues in the form of peat are sloping flat areas and basins, more or less regular rainfall and humid conditions. The basins being permanently filled with water represent an anaerobic system as far as the decomposition of the plant residues is concerned.

In the development of the peat bog, algae and other aquatic plants and products from higher plants, such as pollen and leaves, as well as inorganic dusts, first accumulate, forming the bottom layer of deep peat deposits. This process is very slow and is followed, as soon as the waters become shallow, by a zone of aquatic vegetation, including sedges (*Carex*) and reeds (*Phragmites*). When the level of accumulating material has been raised by the deposit to that of the surrounding country, growth of various herbs and shrubs and of a number of varieties of *Sphagnum* takes place, and, finally, various coniferous trees (*Larix*, *Picea*, *Thuja* and a number of pines) appear; these are followed by deciduous trees. At that stage, the surface of the peat has reached above the level of the bog, so that decomposition begins to keep pace with the accumulation processes. When the water level is suddenly elevated, the trees are destroyed and lower plants adapted to saturated conditions will reappear.

When the growth of peat is not accompanied by a corresponding rise of water level and the surface of the peat reaches far enough above this level, the processes of decomposition keep pace with the processes of peat formation. If such a period is followed again by a rise in water level, a new period of peat accumulation begins and the whole plant

<sup>5</sup> Zailer, V. and Wilk, L. Ztschr. Moork. u. Torfverwert., 5: 40-64, 111-128, 197-260. 1907.

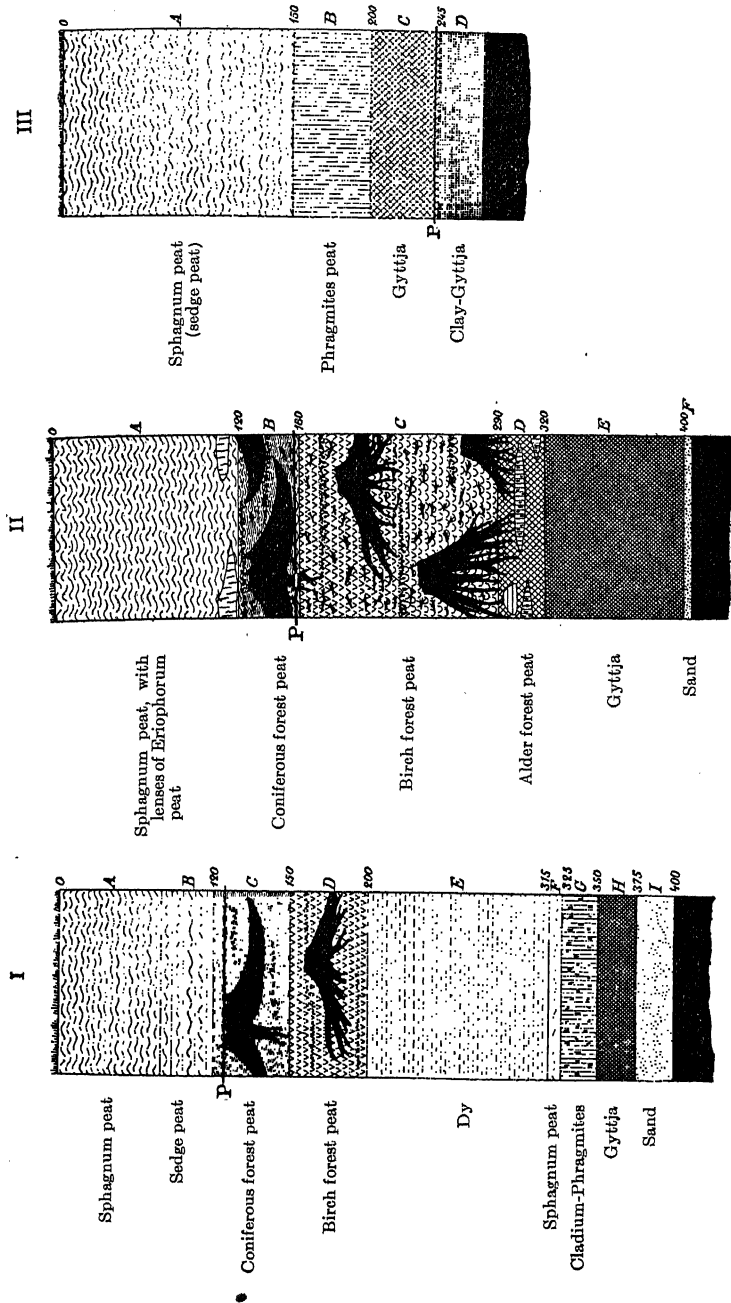


FIG. 58. Several types of peat profiles produced during various climatic periods. P— = limit of occurrence of pine pollen (from von Post).

association may be changed when new conditions are established. Blackened, thoroughly decomposed layers of peat indicate a period of low water level or drought; if such layers are found at different levels of the profile, they indicate that such conditions have occurred at different times during the formation of the peat deposit. The formation of a poorly decomposed, fibrous stratum of plant residues follows periods of prolonged precipitation and rise in water level.

The lower strata of peat formed in filled depressions result from plants growing below the water level and are of much greater fineness than the upper strata which are formed by plants growing above water level; they are practically free from fibrous or woody materials. Built up bogs may consist of the same material for the greater part of their thickness, due to the fact that the ground water level of the deposit may have always been in a definite relation to the surface of the peat.<sup>6</sup>

Species of *Carex* and *Eriophorum* are able to secure all their food from the water and the air for the building of their own substrate. The mass of roots and rhizomes may attain a thickness of several inches and float upon the surface of the water. Sphagnum and shrubs may advance upon this foundation. A gradual progressive submergence of the floating substrate takes place and is accompanied by a gradual disintegration.<sup>7</sup>

*Chemical composition of peat.* The chemistry of peat remained up until the early part of this century, most confusing. The conception of Fröh<sup>8</sup> and others that "the transformation products of plants which are characteristic of peat are the ulmic acids and ulmins, humic acids and humins, as well as the salts of these" dominated the ideas of practically all the investigators who have paid any attention to the chemical nature of peat. The process of "ulmification" was considered as the all important process in peat formation, as shown in Weber's definition. Some understood by this the so-called putrefactive processes involved in peat formation, due to exclusion of air. However, Lamarlière<sup>8a</sup> has shown in 1900 that coniferous wood freshly removed from a peat bed preserves its microscopic structure, but a chemical analysis shows that the cellulose is largely gone and the amorphous residue, after treatment with chlorine, dissolves readily in alkalies.

<sup>6</sup> Davis, C. A. U. S. Bur. Mines, Bul., 38. 1913.

<sup>7</sup> Transeau, E. N. Bot. Gaz., 40: 351-375, 418-448, 1905; 41: 17-42. 1906.

<sup>8</sup> Fröh, J. J. Über Torf und Dopplerite. Eine minerogenetische Studie. Zürich. 1883; Ber. Schweiz. bot. Gesell., 1891, H. 1: 62-79.

<sup>8a</sup> Lamarlière de, L. G. Compt. Rend. Acad. Sci., 131: 511-512. 1900.

No attempt was made, in the study of the origin of peat, to determine whether we are dealing with a single process of transformation or a group of processes. Even Weber assumed that, in the presence of an excess of water, the cellulose molecule of the plant breaks up giving, 1. a small amount of carbon, hydrogen and oxygen, and 2. a colloidal body, which is partly indifferent and partly possesses the nature of acids and its salts. This residual colloidal molecule was known as "ulmin." When air is admitted, the "ulmin" becomes colored dark-brown or black. This "ulmin" molecule was believed to form compounds with nitrogen and phosphorus;<sup>9</sup> a part of the nitrogen was believed to exist in the form of amino acids and acid amides,<sup>10</sup> and a part in the form of animal residues and excreta. The "ulmic acids" were soluble in alkalis, while the "ulmins" dissolve only in strong KOH solutions and could be made soluble by treatment with HNO<sub>3</sub>.<sup>11</sup>

Most of the chemical analyses of peat as well as of peat forming plants were limited to the determination of dry organic matter, moisture, ash and nitrogen content. In many instances, a detailed analysis of the various ash constituents was undertaken; especially of the calcium, magnesium, phosphorus and potassium in the peat. Frequently the sulfur and sodium content were also measured. How little information was gained by such methods of analyses was already recognized by Zailer and Wilk, who said that there are cases on record where the chemical analyses of the ash, calcium and mineral content of a peat correspond to that of a highmoor, while actually the material represented a lowmoor peat, and *vice versa*. The study of the chemical composition of the organic matter, which makes up 60 to 99 per cent of peat, did not, in most cases, proceed further than an elementary analysis, including carbon, hydrogen, oxygen and nitrogen. In some cases, certain organic constituents, especially the ether and alcohol soluble fractions, were separated. Von Feilitzen and Tollens<sup>12</sup> introduced a new angle into the chemistry of peat, by determining the pentosan and cellulose content. The nitrogenous compounds of peat received considerable attention, as shown later.

<sup>9</sup> Tacke, Br. Chemik. Ztg., 21: No. 20. 1897; Landw. Jahrb., 41: 717-754. 1911.

<sup>10</sup> Sestini, F. Landw. Vers. Sta., 51: 153. 1898.

<sup>11</sup> Further studies on the historical aspects of the nature of the organic complexes in peat are given by Baumann, A. and Gully, E. Mitt. Kais. Bayr. Moorkulturanst., 3: 52-123; 4: 31-156. 1909-1910; Oden, 1919 (p. 653).

<sup>12</sup> Von Feilitzen, H. and Tollens, B. Jour. Landw., 46: 7-22. 1898; see K. Hess and W. Komarewsky. Ztschr. angew. Chem., 41: 541-542. 1928; 42: 336-338. 1929.

The results obtained on the organic analysis of peat, when the methods employed were those of the food chemist, were not more promising, so that Dachnowski,<sup>13</sup> after attempting to differentiate peat into digestible and undigestible fractions, came to the conclusion that this method of analysis "does not give sufficiently forceful illustration of the available organic compounds in plant remains now stored as layers of peat."

Only a careful analysis based upon the determination of definite chemical organic complexes present in the peat could yield the desired information. To make a complete analysis of peat, the same procedure can be followed as has been suggested for plant materials (p. 589). Not only can the chemical constituents be expressed in definite quan-

TABLE 84  
*Proximate chemical composition of some typical peats*  
On per cent basis of dry material

PEAT CONSTITUENT	LOW-MOOR PEAT, NEW JERSEY	SAW-GRASS PEAT, FLORIDA	SEDI-MEN- TARY PEAT, FLORIDA	FOREST PEAT, NEW YORK	YOUNG SPHAG- NUM PEAT, GER- MANY	OLD SPHAG- NUM PEAT, GER- MANY	SPHAG- NUM PEAT, MAINE
Ether-soluble portion.....	0.66	2.98	2.97	3.22	3.08	5.73	2.45
Water-soluble portion.....	3.08	1.73	1.07				
Hemicelluloses.....	10.31	6.41	2.19	5.44	16.88	9.08	20.92
Cellulose.....	0	0.28	0	2.68	19.44	12.38	16.32
Lignin and lignin-like complexes..	38.35	46.12	19.33	60.73	34.04	52.50	25.43
Crude protein.....	22.48	23.06	9.00	14.30	5.23	5.78	5.72
Ash.....	13.22	10.00	59.60	3.90	1.72	1.38	1.84
pH.....	5.9	6.2		4.7	4.1	4.2	3.95

titative terms, but this method gives an opportunity to study the changes that have taken place in the various organic complexes of the plant residues, in the process of their transformation into peat. A series of analyses of several typical types of peat are given in table 84.<sup>14</sup> These results show that highmoor peats are rich in fats and waxes, in cellulose and hemicelluloses, but are low in mineral constituents and

<sup>13</sup> Dachnowski, A. P. Jour. Agr. Res., 29: 69-83. 1924.

<sup>14</sup> Waksman, S. A. and Stevens, K. R. Soil Sci., 26: 113-137, 239-251. 1928; 27: 271-281, 389-398. 1929; see also Thiessen, R. and Johnson, R. C. Anal. Ed. Ind. Eng. Chem., 1: 216. 1929; Oden, S. and Lindberg, S. Brennstoff-Chem., 7: No. 11, 1926; Stadnikow, G. and Baryschewa, A. Ibid., 11: 21-23. 1930; Feustel, I. C. and Byers, H. G. U. S. Dept. Agr. Tech. Bul., 214. 1930.

in nitrogenous complexes; they are highly acid in reaction. The low-moor peats are almost free from cellulose, but they are high in mineral constituents and in nitrogenous organic compounds. The sedimentary peats are especially rich in ash. All of the peats are rich in lignin-like complexes, which can be referred to as "peat-lignin."

The concentration of nitrogen, phosphoric acid and potassium in various peats and in certain peat forming plants is reported in table 85, utilizing the results obtained by Minssen.<sup>15</sup>

The peat forming plants are richer in phosphorus and potassium than peat itself. This is true of the other important mineral constituents as well, due to the fact that when the plant dies the easily soluble salts, which are not in organic combination in the plant especially the alkalis (Na, K), are rapidly washed out, and are immediately re-absorbed by the growing plants and used for the building of new plant protoplasm. The living plants are thus able to use a large part of the nutrients required for their new growth from the preceding generation of plants.

Although most of the chemical analyses of peat include the determination of total nitrogen, very little is known concerning the nature of the nitrogenous complexes in peat. The nitrogen content ranges from as low as 0.5 per cent, in the case of certain sphagnum peats (usually not more than 1 per cent), to nearly 4.0 per cent, in the case of certain lowmoor peats. Nearly all this nitrogen is in an organic form and is not readily acted upon by microorganisms in the undrained peat, but is acted upon very slowly when conditions are made aerobic, as by draining the bog. In most of the studies of the so-called "humic acids," the nitrogenous constituents were not considered at all or were merely dismissed as impurities, since the nitrogen did not fit into the various formulae suggested for these hypothetical "acids." Some investigators have even gone so far as to suggest that the nitrogen is present in the "humus" in the form of ammonia or that it is absorbed from the atmosphere and bound as ammonia, although a simple analysis would have convinced them that practically all the nitrogen in the peat is in organic forms. The presence of ammonia can be demonstrated<sup>16</sup> only in highmoor peats where it is produced as a result of the decomposition of some of the nitrogenous compounds. A large part of the nitrogen is present in peat in the form of protein-like complexes, since, on hydrolysis, various amino acids, such as leucin, tyrosin and iso-

<sup>15</sup> Minssen, H. Landw. Jahrb., **44**: 269-330. 1913; **65**, Ergb.: 124-145. 1927.

<sup>16</sup> Logvinova, L. P. Trans. Sci. Inst. Fertil., No. 56, Moskau, 1929.

leucin, are formed.<sup>17</sup> The amide and humin contents of peat are higher, while the total non-basic and basic nitrogen fractions are lower than in proteins of plants and animals. Twenty per cent hydrochloric acid solution will hydrolyze 80 per cent of the nitrogenous compounds in the peat, the other twenty per cent remaining unacted upon.

In addition to cellulose and pentosans, various peats contain also other hemicelluloses; on hydrolysis with dilute inorganic acids, sphagnum peat gives mannose, galactose, laevulose, in addition to pentoses. The presence of cellulose was demonstrated by treating the peat residue, left after extraction with dilute acid, with concentrated sulfuric acid and demonstrating the formation of reducing sugars. The diminution of the cellulose and pentosan content with an increase in depth of peat is largely responsible for the parallel increase in the total carbon content,

TABLE 85  
*Nitrogen and mineral content of different peats and peat forming plants*

TYPE OF MATERIAL	NITROGEN	P <sub>2</sub> O <sub>5</sub>	K
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Young sphagnum peat.....	0.64-0.74	0.03-0.04	0.02-0.03
Phragmites peat.....	2.29-3.23	0.09-0.28	0.04-0.19
Carex peat.....	2.47-2.94	0.14-0.20	0.05-0.06
Pollen peat.....	0.69-1.09	0.03-0.06	0.01-0.16
"Livermud".....	2.01-3.68	0.09-0.28	0.13-0.33
<i>Hylocomium squarrosus</i> .....	1.27	0.44	0.87
<i>Calluna vulgaris</i> .....	1.04	0.23	0.51
<i>Sphagnum medium</i> .....	0.73	0.09	0.23

since these substances are low in carbon, while the waxy materials ("bitumen") and the lignin, which have a much higher carbon content and are more resistant to decomposition, increase with depth.

Keppeler<sup>18</sup> suggested to divide peat into two fractions, based upon the reactivity of the constituents with 72 per cent sulfuric acid. This treatment was found to change all the cellulose and hemicelluloses to glucose. That part of the peat which was unacted upon by the acid can be considered as lignin or peat-lignin, or humus-lignin. It was suggested to use this method for following the course of peat formation

<sup>17</sup> Jodidi, S. L. Jour. Amer. Chem. Soc., **32**: 396-410. 1910; Robinson, C. S. Mich. Agr. Exp. Sta., Tech. Bul., **7**. 1911; **35**. 1917; Morrow, C. A. and Gortner, R. A. Soil Sci., **3**: 297-331. 1917.

<sup>18</sup> Keppeler, G. Jour. Landw., **68**: 43-70. 1920; Brennstoff-Chem., **2**: 215. 1921.

from the original plants. The reducing sugars obtained by this method from *Sphagnum cuspidatum* were 68.5 to 71.4 per cent of the total plant material and from *Sp. medium*, 61.5 to 64.5 per cent. By determining the ratio between the reducing sugar formed on treating the particular peat with acid to the sugar formed from the original plant material similarly treated, an index of the degree of peat formation was obtained. This method is thus based upon the fact that, with the progress of decomposition of plant residues, there is a parallel disappearance of the cellulose and hemicellulose fractions. In the case of highmoor peats, the reducing power of the original *Sphagnum* plants can be taken as 68. The degree of decomposition of peat can, therefore, be calculated from the following formula:

$$\left(100 - \frac{\text{reduction of hydrolyzed peat} \times 100}{68}\right)$$

A young peat, with a total reduction of 34.7 to 57.3 per cent, gave a degree of decomposition of 49.0 to 15.7 per cent. With an increase in depth of peat, there is a decrease in the total reduction and an increase in the degree of decomposition.

The amount of residue left after treatment with concentrated sulfuric acid (the ash-free lignin) is taken as an index of "peat formation." The "index of peat formation" is fairly comparable to the degree of decomposition.

*Origin of peat.* As pointed out above, the process of peat formation has commonly been considered as one of very simple transformation; the assumption was thereby made that all plant residues falling under water remain preserved from any action of microorganisms and undergo only a weak hydrolysis. In some cases, the chemical constituents of the plant remains were believed to undergo in the bog a certain slow decomposition, being thereby gradually transformed into a dark brown mass, usually referred to as "humus" and believed to consist of "humic acids."

Potonié<sup>19</sup> described the process of peat formation as one of "self-decomposition," believed to represent the last phase of oxidation, decomposition and fermentation processes. Oden<sup>20</sup> spoke of the rôle of atmospheric agencies in peat formation. One of the earlier students of

<sup>19</sup> Potonié, H. Die rezenten Kaustobiolithe und ihre Lagerstätten. Pt. I. Die Sapropelithe. 1908. Pt. II u. III. Die Humudbildungen. Abhandl. Königl. Preusz. Geolog. Landwsanst. H. 55. Berlin. 1911-1912.

<sup>20</sup> Oden, S. Kolloidchem. Beih., 11: 75-260. 1919.



peat, Früh, considered peat formation as a result of a slow breakdown of plant constituents, at a low temperature and in the absence of oxygen, with an inner oxidation and the liberation of water; the action of bacteria was considered to be of no importance in this connection. Soper and Osborn<sup>21</sup> explained the origin of peat from cellulose by a process of carbonization, whereby the carbohydrate loses hydrogen and oxygen in the form of water and carbon and hydrogen in the form of methane. To sum up this attitude towards the nature of the processes involved in peat formation, it is sufficient to cite Bersch,<sup>22</sup> who stated that "in contradistinction to the processes of decay (Verwesung) of organic matter, which take place under aerobic conditions and which are brought about by microorganisms, the processes of peat formation (Vertorfung) are purely chemical in nature."

Since cellulose forms the largest single group of constituents in plant remains, it was frequently assumed that it gives rise to peat, either by the removal of oxygen and water, or by its transformation first into oxycelluloses. The fact that the cellulose content tends to diminish in peat with age and may even disappear completely seemed to lend support to this hypothesis.

More recently, however, the work of Fischer and Schrader<sup>23</sup> and others focussed attention upon the rôle of lignins in peat formation. According to this theory, the celluloses of the plant residues are decomposed by microorganisms under the anaerobic conditions prevailing in peat bogs, giving rise to gaseous products ( $\text{CH}_4$ ,  $\text{CO}_2$ ), water and organic acids. The lignin, on the other hand, is more resistant to decomposition by microorganisms and, therefore, accumulates. With the advance of decomposition, the methoxyl groups in the lignin complex are saponified and changed to hydroxyl groups, with the result that a phenol-like, alkali-soluble, body, namely "humic acid" is formed; this complex may later be transformed into "humins," which is insoluble in alkalies.

According to Bergius,<sup>24</sup> both cellulose and lignin are capable of giving rise to peat and to coal, under the proper conditions of temperature and pressure. Marcusson<sup>25</sup> came to the conclusion that cellulose is changed by hydrolysis and oxidation to oxycellulose and pectin; the

<sup>21</sup> Soper, E. K. and Osborn, C. C. U. S. Geol. Survey, Bul., 728. 1922.

<sup>22</sup> Bersch, W. Handbuch der Moorkultur. Wien. 1909.

<sup>23</sup> Fischer, Fr. Naturwiss., 9: 958-965. 1921; Fischer, Fr. and Schrader, H. Brennstoff-Chem., 3: 65-72. 1922; Stadnikoff, G. Neuere Torfchemie. Steinkopff, Leipzig. 1930; Fuchs, W. Die Chemie der Kohle. J. Springer. Berlin. 1931.

<sup>24</sup> Bergius, F. Naturwiss., 16: 1-11. 1928.

<sup>25</sup> Marcusson, J. Ztschr. Angew. Chem., 39: 898-900; 40: 48, 1104-1106. 1927.

glucuronic acid compound of these substances is transformed, on heating, to "humic acid;" this mechanism is believed to be responsible for the formation of the peat and coal. These theories are entirely hypothetical in nature and are not based upon sufficient experimental evidence, comparable to processes taking place under natural conditions.

Wiegmann<sup>26</sup> was the first to attempt a solution of the problem of the origin of peat by chemical analysis of the peat-forming plants and of young and old peat. He placed peat forming plants in a pit, covered them with water and pressed them down with a heavy cover for a period of 6 to 12 months. Senft<sup>27</sup> observed that, under such conditions, the plants are transformed into brown, humus-like substances. Fröh placed a mixture of plants of *Sphagnum* with *Erioph. vaginatum* or with algae in tall cylinders; the plants were covered with water, then with a 1.5 centimeter layer of oil and allowed to remain at 10°C.; the cylinders were opened after 6 months. The formation of the so-called "ulmic acids" and "ulmins," "humic acids" and "humins," as well as of salts of these acids was noted. A solution of 5 per cent KOH was used for the separation of these complexes. Fröh recorded that cellulose "ulmifies" quite completely, and the more readily the younger the plant. Lignin containing substances decomposed with greater difficulty, while mosses were found to be quite resistant to decomposition; wax-like compounds were found to remain largely unchanged, especially in sphagnum bogs. As to the part played by microorganisms in peat formation, Fröh suggested in 1879 that the tissues of peat forming plants disappear by a "peaty fermentation," with the evolution of gas. No bacteria active in the "humification" process could be found.<sup>28</sup>

Klason<sup>29</sup> stated in 1893 that "the process whereby dead plants change into the first stage of peat must consist in the fermentation of the carbohydrates; when this takes place under water, in the absence of air the process must stop here, the active microorganisms being unable to ferment the other plant constituents. Peat actually consists of lignin-like substances of plants in a more or less unchanged condition." It is generally agreed now that with an increase in the decomposition of peat in highmoors, a decrease in the content of cellulose, pentosans

<sup>26</sup> Wiegmann. Ueber die Entstehung, Bildung und das Wesen des Torfes. Braunschweig. 1837.

<sup>27</sup> Senft. Die Humus-, Marsch- und Torfbildung. Leipzig. 1862.

<sup>28</sup> See, for an early conception of origin of peat, Lesquerreux, L. Quelques recherches sur les marais tourbeux en général. Neuchâtel. 1844.

<sup>29</sup> Cited by Oden, S. and Lindberg, S. Brennstoff-Chem., 7: 165. 1926.

and other carbohydrates, and an increase in the content of extractives obtained by means of organic solvents, of lignin and "humic" substances take place.<sup>30</sup>

The theory presented by Fischer, concerning the rôle of lignin in the formation of peat and coal overlooks four very important facts:

1. Lignin is not absolutely resistant to decomposition under aerobic conditions, as shown by the continuous disappearance of organic matter in cultivated soils; this process is carried out by certain bacteria, actinomyces, and higher fungi;<sup>31</sup> under certain conditions, various Basidiomycetes are capable of decomposing the lignin as rapidly as they do the cellulose, as in the destruction of wood by certain fungi.

2. The high content of nitrogen in certain kinds of peat, especially in the lowmoor and sedimentary peats, where the cellulose and hemicelluloses have undergone marked decomposition and have practically disappeared; no consideration is given to the source and nature of these nitrogenous compounds, which may make up 25 to 30 per cent of the total organic complexes in the peat; no attempt is made to explain the rôle of this nitrogen in peat formation.

3. This theory does not consider the fact that certain types of peat, like the lowmoors, are practically free from cellulose, while others, like the highmoors or sphagnum peats, are rich in cellulose; it does not account either for the high hemicellulose content in nearly all types of peat.

4. Lignin forms at most only 40-50 per cent of the peat material; at best, the theory proposed by Fischer only confirms and develops further the previous observations of other investigators that lignin is more resistant to decomposition than the other plant constituents and, therefore, accumulates in the process of degradation of plant materials by microorganisms.

In the decomposition of reeds and sedges, trees and other similar plants, the cellulose and pentosans undergo rapid attack by fungi and aerobic bacteria at the surface of the bog, and later by anaerobic bacteria, when the plant remains are immersed in water. This is made especially easy, due to the presence of mineral salts and available nitrogen as well

<sup>30</sup> Maliutin, B. N. *Verhandl. wiss. Torfforsch. Inst. Moskau.* 1928, p. 58-74; Thiessen, G. and Engelder, C. J. *Jour. Ind. Engin. Chem.*, **22**: 1131-1133. 1930; Thiessen, R. and Johnson, R. C. *Ibid, Anal. Ed.*, **1**: 216. 1929; Fuchs, W. *Brennstoff-Chem.*, **11**: 106-112. 1930.

<sup>31</sup> Phillips, M., Weihe, H. D. and Smith, N. R. *Soil Sci.*, **30**: 383-390. 1930; Tenney and Waksman, 1929 (p. 600).

as due to a favorable reaction, both phenomena being characteristic of lowmoor peats. The cellulose and pentosans gradually disappear, and the energy thus made available allows the microorganisms to synthesize considerable cell substance rich in nitrogenous organic complexes, referred here collectively as protein. As a result of these synthesizing activities of the microorganisms there is a gradual accumulation of proteins and other organic nitrogenous complexes, parallel to the decomposition of the cellulose and hemicelluloses, as shown elsewhere (p. 617).

In the case of the highmoor peats, the cellulose and hemicelluloses of the Sphagnum plants are much more resistant to decomposition than in the herbaceous plants, while the high acidity of the bogs and the lack of sufficient mineral nutrients make conditions unfavorable for the growth of many groups of microorganisms, especially under anaerobic conditions. On the other hand, the amides and certain other nitrogenous compounds of these plants decompose readily, even under the high acid conditions, liberating the nitrogen in the form of ammonia. This may either remain in an adsorbed state, the acid conditions preventing the activities of the nitrifying bacteria, or it is immediately utilized by the growing part of the plant, which is thus able to obtain its nitrogen from the dead parts which undergo partial decomposition. This phenomenon accounts for the high ammonia content of sphagnum peat and for its comparatively low protein content, or abundance of organic nitrogenous complexes.

The marked difference in the chemical composition of the several types of peat is thus explained by the nature of the chemical complexes in the original plant materials, the nature of the decomposition processes carried out by microorganisms, and the conditions under which decomposition is taking place.

*Bacterial and fungus population of peat.* A number of the earlier students of peat were convinced that either bacteria and other microorganisms are totally absent in peat bogs, or at least have nothing to do with the formation of peat from the plant remains. Later, definite evidence has been submitted that large numbers of microorganisms are found in peat, their nature and abundance depending largely upon the depth of the particular peat layer, the nature of the peat and its physical and chemical properties. This is brought out in tables 86 and 87. Not only are numerous bacteria found at various depths of both lowmoor and highmoor peats, but some of them, namely the anaerobic forms, actually increase with depth. Nitrifying bacteria, aerobic

cellulose decomposing bacteria and actinomyces are present only in lowmoor peats, either near the surface or not far from it. Fungi are also present, but only in the upper layers of the bog.

Begak<sup>32</sup> demonstrated that, by the use of meat peptone agar, sphagnum peat may only show about 4,000 cells per gram of the upper 10 cm. layer; these numbers increase to 90,300 per gram at a depth of 25-30 cm., then gradually diminish, until the "Grenzhorizont" is reached, when they begin to increase again. However, by the use of the direct

TABLE 86

*Occurrence of microorganisms at different depths of a lowmoor peat profile at Newton, N. J.*

On the basis of fresh peat material

DEPTH OF SAMPLE	pH	MOISTURE CON- TENT	BACTERIA (AEROBIC AND FACUL- TATIVE ANAEROBIC) AND ACTI- NOMYCES PER GRAM	ACTINO- MYCES	FUNGI PER GRAM	AEROBIC* CELLU- LOSE DECOM- POSING BACTERIA	NITRI- FYING BAC- TERIA*	ANAE- ROBIC BACTERIA*
cm.		per cent		per cent				
Surface	5.9	61.1	6,000,000	90	105,000	++	+++	+
30	6.0	72.5	350,000	40	250	+	++	++
45	6.2	82.3	450,000	25	175	0	++	++
60	6.3	87.5	40,000	20	150	0	+	++
75	6.3	87.1	35,000	25	33	0	+	++
90	6.4	80.8	20,000	15	0	0	0	++
120	6.7	83.6	100,000	2	0	0	0	+++
150	6.8	84.5	500,000	0	0	0	0	++++
165	8.0	64.8	200,000	0	0	0	0	++++
Clay bottom								

\* + designates a few; ++, a fair number; +++, abundance of organisms; +++++, numerous (about 25,000 or more per one gram of material).

microscopic method of soil examination, 323 to 715 millions of bacteria were found per gram of moist material, amounting to three to seven billions of cells in each gram of dry sphagnum peat. These bacteria were made up of 1.25 per cent large rods, 60.0 per cent short rods, 20 per cent cocci, and about 20 per cent thick rods and small cocci. This abundant microflora of bacteria as well as an abundant development of fungi were believed to prove that these are the active agents in the decomposition processes taking place in peat. The acidity of the

<sup>32</sup> Begak, D. A. *Pedology*, 21: 64-75. 1926.

sphagnum was equivalent to pH 3.3 to 4.2. The bacteria were found to be well adapted to this high acidity.

Ritter<sup>33</sup> also criticized the Koch plate method as altogether unsuitable for giving any idea concerning the abundance of the bacterial population in peats. The nitrifying, anaerobic, nitrogen-fixing and other organisms do not develop at all on this medium. The colloidal nature of the peat makes an even distribution of the bacteria much more difficult. Ritter's results have shown that the cell content (as determined by the plate method) of uncultivated highmoor peats is usually very low; young or little decomposed peat is always poorer in bacterial cells than well

TABLE 87

*Occurrence of microorganisms at different depths of a highmoor peat profile from Maine<sup>34</sup>*

On basis of fresh material

DEPTH OF SAMPLE	pH	MOISTURE CONTENT	AEROBIC AND FACULTATIVE ANAEROBIC BACTERIA PER GRAM	ACID-RESISTING ANAEROBIC BACTERIA
<i>cm.</i>		<i>per cent</i>		
Surface layer			250,000	0
7.5-20	4.05	92.7	100,000	+
20-30	3.95	92.6	125,000	+
30-45	3.85	92.6	1,600,000	+
45-60	3.86	92.9	2,500,000	++
60-75	3.73	93.6	1,500,000	++
120-150	3.90	93.6	500,000	+++
175-210	4.47	93.4	750,000	++
450-480	4.71	92.4	800,000	+++
540-570	5.18	92.2	2,000,000	++++

decomposed peat. Peat cultivated for a long time, limed and fertilized was found to contain incomparably more bacteria than untreated peat; when peat is well decomposed, it may contain more bacteria than mineral soils. An untreated peat receiving only an application of lime showed a relatively moderate increase in bacterial cells. Lowmoor peats contained in all cases more bacteria than highmoor peats, even in an uncultivated state. As in mineral soils, the numbers of bacteria were found to decrease rapidly with depth, but they are still present even at a depth of 50 cm., when infection was fully excluded. A dif-

<sup>33</sup> Ritter, G. A. Intern. Mitt. Bodenk., 2: 411-428. 1912.

<sup>34</sup> Waksman, S. A. and Stevens, K. R. Soil Sci., 28: 315-340. 1929.

ference in the composition of the medium could not show any great variation in the numbers of bacteria.

Microorganisms in general play an active rôle in four distinct phases in the chemical transformations involved in peat formation and peat decomposition:

1. Those microorganisms which are active during the first stages of decomposition of the plant residues, either before these have become submerged or even after they have become covered with water; in these processes of disintegration some of the chemical constituents of the plants rapidly disappear. Certain groups of organisms, largely fungi on the surface of the bog and bacteria below the surface of the anaerobic medium, bring about the decomposition of the sugars, of certain hemicelluloses, of the cellulose, and of some of the proteins and their derivatives. The carbon dioxide and ammonia thereby liberated are used immediately by the growing plants; this process is of great importance in bogs poor in nutrients, as in the case of the sphagnum peats.

2. Microorganisms active in the various layers of the peat profile, long after the initial stages of decomposition have passed. Here we are dealing almost entirely with facultative and obligate anaerobic bacteria, and possibly certain anaerobic actinomycetes. The pockets of gas rich in hydrogen and methane, as well as certain putrefactive odors, frequently found at various depths of the peat profile are due to the gradual decomposition of the cellulose and other complexes by these bacteria.

3. Microorganisms active in the decomposition of the organic complexes in the peat, after the bog is drained, as in the case of lowmoor and sedimentary peats, or drained and limed as in the case of the highmoor peats. Here we are dealing with various fungi, aerobic bacteria and actinomycetes decomposing the resistant peat complexes, with the liberation of large quantities of ammonia; this is rapidly changed to nitrates by nitrifying bacteria. These nitrates may accumulate in quite considerable quantities in the surface layers of the drained peat. Frequently and under certain conditions, especially in highmoor bogs receiving excessive amounts of lime, nitrate reducing bacteria may become active; this leads to losses of nitrogen to the atmosphere.

4. Microorganisms that have contributed directly by their cell substance to the formation of certain peats. This is true especially of sedimentary or allochthonous peats in which fungus spores and mycelium, as well as various algae and bacteria, may be quite abundant.

Peat is formed because of the fact that the saturation of the bog with

water produces anaerobic conditions. This does not prevent the growth of plants adapted to that environment, but it does prevent the growth of fungi, actinomyces and aerobic bacteria which would be capable of decomposing the plant residues. The obligate and facultative anaerobic bacteria favored by these conditions are capable of attacking only some of the organic complexes, leaving the other constituents to accumulate and thus give origin to peat. Lignin, either as such or in a modified form, certain nitrogenous substances and various hemicelluloses predominate in lowmoor peats, while waxes ("bitumens"), cellulose and certain hemicelluloses, and lignin-like complexes predominate in the highmoor peat formations.

Natural sphagnum peats possess low microbiological activities. Drained and cultivated peats become very active, as shown by the evolution of  $\text{CO}_2$  and nitrate formation. The nitrogen becomes readily mineralized in cultivated peats. The nitrogen-fixing capacity is rather weak and is largely of the anaerobic type.<sup>35</sup>

Lowmoor peats contain at the surface and to some depth as well, a considerable number of actinomyces. The number of these organisms increases when the bog is drained and cultivated. It is believed that actinomyces take an active part in the decomposition of some of the resistant complexes in the peat and lead to the liberation of the nitrogen in an available form. Lowmoor peats also possess a vigorous flora of nitrifying and cellulose-decomposing bacteria. These are largely responsible for the abundant formation of nitrates in the upper layers of the peat and the almost complete lack of cellulose. When a lowmoor peat is moistened with an ammoniacal solution, it forms a very excellent medium for the formation of nitrates, provided the compost is properly aerated and the reaction is not allowed to become too acid. Fungi are found to be abundant in lowmoor peats, but only at the very surface of the undrained bog.

Highmoor peats, however, due to their high acidity are free from nitrifying and aerobic cellulose decomposing bacteria as well as actinomyces. They contain a highly specific bacterial flora, partly aerobic, partly facultative anaerobic, and partly obligate anaerobic, which grows readily at pH 4.0, a phenomenon not observed commonly among soil bacteria.<sup>36</sup> *Azotobacter* is absent in highmoor peats, but *Bac. amylobacter* is present.

<sup>35</sup> Cholkin, I. C. Bull. Bur. Agr. Microb. Leningrad, 3: 131-152. 1928.

<sup>36</sup> Christensen, H. R. Centrbl. Bakt. II, 37: 414-631. 1913; Drewes, K. Centrbl. Bakt. II, 76: 114-121. 1928.



Omeliansky<sup>37</sup> found in lake mud or sedimentary peat formations the presence of the following groups of bacteria: proteolytic, denitrifying, anaerobic nitrogen-fixing, pectin fermenting, aerobic cellulose decomposing, anaerobic cellulose decomposing and fat splitting. He came to the conclusion that sedimentary peat (Sapropel) represents a medium in which various bacterial processes take place energetically, leading to the decomposition of proteins, carbohydrates and fats.

*Microfauna of peat bogs.* The microfauna of peat bogs is represented by the following groups:

Rhizopods. Naked amoebae are rare, while testaceous rhizopods are abundant. Heliozoa are not as abundant as the first group, some finding optimum conditions in the highmoor and others in the forest peats. Ciliates are very limited in highmoors, but are more abundant in the other types of peat. Hydrozoa are absent in highmoors but are found in forest peats. Turbellaria occur to a limited extent in the highmoor and to a greater extent in the forest peats. The same is true of the rotatorians, nematodes and other invertebrates. These are represented in highmoors only by very few species. The fauna in the bogs is largely controlled by the water supply and nutrition.

It is of special interest to call attention to the specific associations of the rhizopods in different peats. An abundant fauna<sup>38</sup> of *Amphitrema* (*A. flavum* and *A. writhianum*) has been found in fully developed highmoors. These may be missing in non-fully developed peats, but *Hyalosphenia papilio* and *H. elegans* are common to both. A study of a number of samples of sphagnum peat revealed the following groups of rhizopoda: 1. Species of *Diffugia*, *Centropyxis*, *Arcella*, *Nebela*, *Euglypha*, *Assulina*, *Corythion* and *Trinema*. This is recognized as of the forest-moss type association, although distinctly sphagnum types, such as *Nebela militaris*, are also found here. 2. Some species found in group 1 and, in addition, *Hyalosphenia papilio* and *H. elegans*; this group comprises a more constant and regular fauna, known as the *Hyalosphenia* type. 3. In addition to the above organisms, *Amphitrema* species are also present; fauna of this type is richest in kinds and is known as the *Amphitrema* type. 4. *Quadrula symmetrica* predominates, as well as *Cyphoderia* and *Nebela* species. The types of rhizopod-associations present in peat bogs are thus found to correspond to definite botanico-geological groups.

<sup>37</sup> Omeliansky, W. L. Russ. Jour. Microbiol., 4: 186-195. 1917.

<sup>38</sup> Heinis, Fr. Arch. Hydrobiol., 5. 1910; Harnisch, O. Biol. Zentrbl., 44: 110-127. 1924; Harnisch, O. Die Biologie der Moore. Schweizerbart. Stuttgart. 1929.

*Microbiological processes in peat soils, drained and cultivated.* When a peat bog is drained, a marked change takes place in its microbiological population. When the anaerobic conditions are made aerobic, large numbers of fungi, actinomyces and aerobic bacteria, especially the nitrifying organisms, develop. To favor the growth of most of these bacteria, a highmoor peat must first be limed. The low bacterial content of highmoor peats was explained by Fabricius and von Feilitzen<sup>39</sup> as due to the high acidity of the latter; drainage alone did not bring about any marked bacterial multiplication; liming, cultivation, manuring and treatment with sand brought about considerable development of bacteria. Well manured and cultivated highmoor peat soil, however, contained as many bacteria as lowmoor peat soil under the same conditions. Soil temperature was also found to influence appreciably bacterial development, as shown in the following summary:

<i>Treatment of peat</i>	<i>Numbers of bacteria per gram, by the plate method</i>
Raw, uncultivated peat.....	138,500
Drained, but not cultivated peat.....	200,300
Freshly cultivated, highmoor peat, treated with sand and lime.....	6,900,400
Long cultivated highmoor peat treated with sand, lime and manure.....	6,224,500
Same, under fallow.....	7,801,000
Same, with oats grown.....	7,175,000

When a highmoor peat is moderately limed, no nitrification will take place within the first year; an excessive addition of lime will bring about rapid development of nitrifying bacteria. A moderate amount of lime will increase crop productivity, but an excess of lime above certain limits will not result in such high crop yields. This was explained<sup>40</sup> by the fact that the increase in the amount of lime added to the peat stimulates the development of denitrifying bacteria, which may lead to considerable losses of the nitrogen.

Although the Remy solution method was used by Arnd<sup>40</sup> for comparing the activities of microorganisms in different layers of a highmoor peat under different treatments, a method not very satisfactory for measuring the activities of the microbial population in normal field soils, marked differences were obtained: surface material from an untreated

<sup>39</sup> Fabricius, O. and von Feilitzen, H. *Centrbl. Bakt.* II, 14: 161-168. 1905.

<sup>40</sup> Arnd, Th. *Centrbl. Bakt.* II, 45: 554-574; *Landw. Jahrb.*, 51: 297-328. 1918.

peat bog produced, in six days, from a 50 cc. peptone solution, 3.1 mgm. of nitrogen as ammonia, while peat from a depth of 20–40 cm. produced

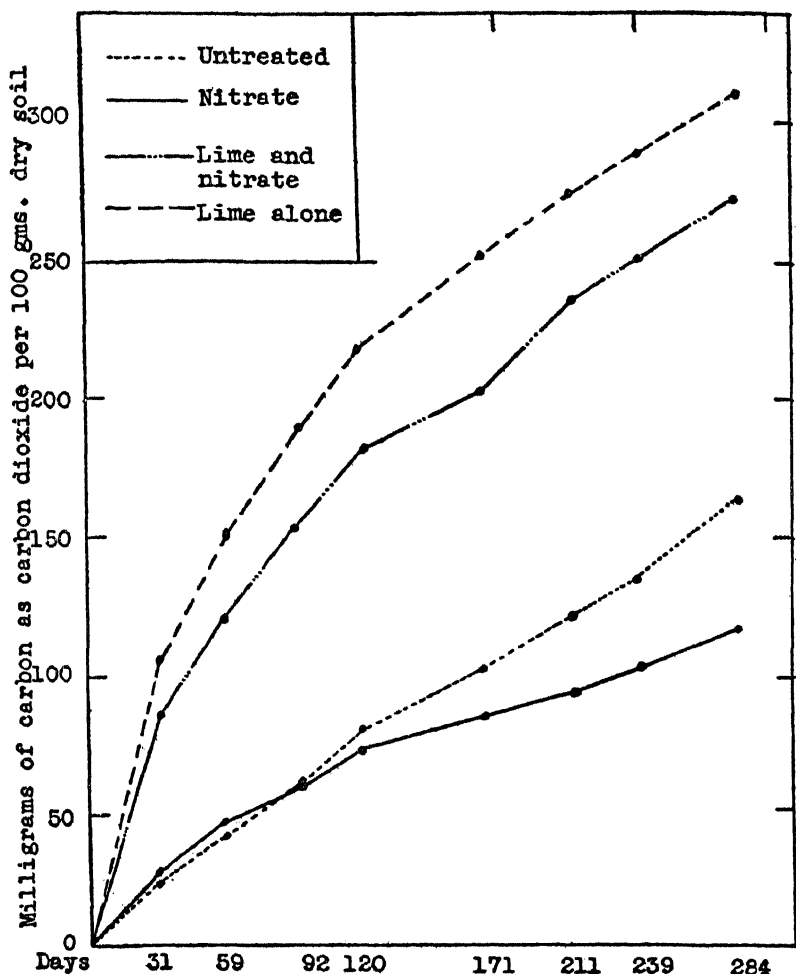


FIG. 59. Influence of liming and addition of available nitrogen upon the decomposition of organic matter in a highmoor peat bog, as shown by the evolution of CO<sub>2</sub> (from Shunk).

only 0.8 mgm. The same peat, when drained, limed and fertilized produced 8.2–10.3 mgm.; when the same peat was also manured, 27.8 mgm. of ammonia nitrogen were liberated under the same conditions.

According to Tacke,<sup>41</sup> the addition of stable manure to peat has a favorable effect in stimulating the liberation of nutrients. The activities of the microorganisms were measured by adding 500 mgm. of nitrogen in the form of peat to 500 gm. of sandy soil; this was adjusted to optimum moisture content and incubated for 70 days at 22°C. Manured peat liberated 20.5–22.2 mgm. of nitrogen as nitrate, while unmanured peat liberated only 6.3 mgm. of nitrate nitrogen. The favorable action of manure was ascribed to the stimulating effect upon the activities of microorganisms in the peat.

TABLE 88

*Influence of nitrate and lime upon bacterial development and decomposition of organic matter in soil taken from a peat bog*

On basis of 100 gm. of soil and 9 months incubation

TREATMENT OF PEAT	pH	NUMBERS OF BACTERIA BY PLATE METHOD, PER 1 GRAM	NH <sub>3</sub> -N	NO <sub>3</sub> -N	TOTAL NITROGEN MADE AVAILABLE	INCREASE DUE TO TREATMENT
			mgm.	mgm.	mgm.	mgm.
No treatment.....	5.2	2,560,000	4.7	0	4.7	0
15.6 mgm. nitrogen added as sodium nitrate.....	4.8	5,034,000	11.2	11.7	22.9	2.6
0.5 per cent CaCO <sub>3</sub> added.....	7.3	118,050,000	0.7	13.3	14.0	9.3
15.6 mgm. nitrogen as sodium nitrate + 0.5 per cent CaCO <sub>3</sub> added.....	7.3	111,485,000	0.6	24.7	25.3	5.0

The influence of lime and additional nitrogen upon the decomposition of peat in a highmoor bog can be measured by the evolution of CO<sub>2</sub>, as shown in fig. 59. The addition of nitrate to such soil had no effect upon the development of bacteria, or decomposition of the organic matter, as measured by the evolution of CO<sub>2</sub> or liberation of available nitrogen (table 88).<sup>42</sup>

It is of interest to note here that certain specific physiological diseases of plants grown in certain types of peat or in inorganic soils with a peat horizon may be due to a toxic substance which is present in the peat. This substance is soluble in alcohol and is carried over in

<sup>41</sup> Tacke, Br. Jahrb. Moork., 13: 27–32. 1924.

<sup>42</sup> Shunk, I. V. Soil Sci., 27: 283–304. 1929.

the distillate at 100°C; it is precipitated by copper. As a result of this phenomenon, the application of small quantities of copper (50 to 100 pounds per acre) has been found to exert a checking effect upon the disease.<sup>43</sup>

<sup>43</sup> Smith, W. S. Thesis. Wageningen. 1927. For a detailed discussion of the physical properties and agricultural utilization of peat, see Br. Tacke. Die naturwissenschaftlichen Grundlagen der Moorkultur. P. Parey. Berlin. 1929; Stadnikoff, 1930 (p. 654).

## CHAPTER XXVII

### DECOMPOSITION OF ORGANIC MATTER IN FOREST SOILS AND MICRO-ORGANISMS CONCERNED

*Nature of organic matter in forest soils.* Forest soils, just as peat soils, possess microbial populations which are distinct from those found in field and garden soils. They are also characterized by marked differences in the nature of the organic matter and mechanism of its decomposition. This is due largely to the fact that in forest soils the organic matter remains on the surface where it undergoes decomposition largely in an organic medium, at least during the first stages. However, while in peat, after the early periods of decomposition, further transformation of the organic matter is considerably delayed due to the prevailing anaerobic conditions, in forest soils, which usually represent good aerobic systems, decomposition may go on very rapidly and nearly to completion. The nature and chemical composition of the plant residues, combined with the conditions under which decomposition is taking place, determine the nature of the forest soil produced.

The specific nature of the organic matter found in forest soils is utilized for the separation of these soils into three types:<sup>1</sup> 1. "Mull" and "mull-soils," when the plant residues can no longer be distinguished, due to previous decomposition; the organic matter is well mixed with the inorganic constituents of the soil; insects and worms are believed to play a predominant rôle in the formation of this type. 2. "Leaf mold" or "Moder" soils, where the microscopic structure of the plant residues is still recognized; the mixture of the organic matter with the inorganic part of the soil is incomplete. 3. "Dry peat," in which the plant residues, of several years accumulation and with a well distinguished structure, remain on the surface of the soil, without any appreciable mixture with the soil itself. More frequently two types of organic

<sup>1</sup> Emeis, C. Waldbauliche Forschungen und Betrachtungen. Berlin. 1875; Müller, P. E. Nogle Undersøgelser af Skovjord. Copenhagen. 1878; Vater, H. Mitt. Sächs. Versanst. Tharandt, 3: 129-181. 1928; Kühn, G. Chemische Untersuchungen des Trockentorfs. Hannover. 1929; Hackmann, G. Forstarchiv, 5: 321-326. 1929; Romell, L. C. and Heiberg, S. Ecology, 12: 567-608. 1931.

matter formation are recognized in place of the "dry peat," namely the "raw-humus" and "surface peat" types.

Emesis recognized in 1875 three classes of forest soil, one of which corresponds to a true mull and the other two to raw humus. In 1878, P. E. Müller distinguished two types of humus formation in forest soils ("mull" and "mor"), which are well characterized biologically, chemically and silviculturally, and which in their turn influence the type of soil formation. The "mull" soil represented a loose, friable mass with a crumbly structure, the organic matter being intimately mixed with the mineral soil, and is inhabited by large earthworms. The "mor" soil consisted of a humus layer, high in organic matter and lying on the surface of the mineral soil, thus corresponding to the modern "raw humus" soil. The local designation "duff" was adopted by Romell and Heiberg for the "raw humus" soil formation. These types of soil do not remain, however, constant, but are markedly influenced by climatic factors and silvicultural practice.<sup>1a</sup>

Hesselman<sup>2</sup> distinguished 3 distinct layers of organic matter formations on the surface of the forest soil, consisting of fresh litter and of decomposing organic matter: 1. "Förna," or the surface layer of unchanged plant and animal residues; 2. "Förmultningsskiktet," "Vermoderungsschicht" in German, or that layer which consists largely of the plant residues undergoing active decomposition (F layer); 3. "Humusämneskiktet" or "Humusstoffschicht," or the layer which consists of newly formed amorphous organic substances, in which the structure is no longer discernible (H layer).

On the basis of the activities of various microorganisms bringing about the decomposition of organic matter in forest soils, Falck<sup>3</sup> differentiated two distinct processes, namely mycocriny and anthracriny.

*Mycocriny* comprises the decomposition of plant products exclusively by fungi; a definite flora of wood-destroying fungi (Basidiomycetes), accompanied by various molds and myxomycetes, can bring about the complete destruction of the plant residues. The total carbon is changed partly to CO<sub>2</sub> and is partly used for the synthesis of the fungus mycelium, while the nitrogen and minerals of the plant products are largely transformed into fungus mycelium and spores. The synthesized mycelium and spores are an excellent forest fertilizer, and form the "acid humus" or "raw-humus" soil. In *anthracriny*, the process of decomposi-

<sup>1a</sup> Fisher, R. T. Ecology, 9: 6-11. 1928; Harvard Forest, Bul. 15, 1930.

<sup>2</sup> Hesselman, H. Meddel. Stat. Skogsförsöks. H. 22, No. 5, 1926.

<sup>3</sup> Falck, R. Mykologische Untersuchungen und Berichte, vol. 2, Geb. Cassel. 1923.

tion of forest products by fungi is interrupted by various soil insects, larvae and worms that consume the mixture of plant residues, which has been previously thoroughly attacked by the fungi, and the fungus mycelium itself; these animals excrete later dark, humus-like, residues. This material can now undergo a process of nitrification, and the soil thus formed belongs to the "mull" type.

According to Falck, a good forest soil consists of four layers: (1) an upper layer of unchanged residues, heavier in the autumn when the leaves have fallen; (2) a fungus layer especially active in spring and early summer; (3) a true humus layer consisting of dark-colored insect excreta, mixed with decomposed and powdered leaf residue rich in bacteria and animals; and (4) the first layer of mineral soil, containing extractives of the upper layer washed down by rain water. In the case of mycorrhizy predominating, there is no dark muck layer, the unchanged mineral soil following soon after the fungus layer. In some cases, the fungus layer is at a minimum and the muck layer is more extensive. In the second layer the plant residues are all surrounded with the fungus mycelium (the abundance of the fungi under these conditions cannot be determined by the plate method, since the mycelium extends throughout the soil, representing one individual. For such studies the direct microscopic method should be used). The fourth layer is ramified with the roots of trees, which may also penetrate into layers two and three. The two processes of decomposition of organic matter result in a constant evolution of carbon dioxide and later in a continuous formation of nitrate. The first is rapid at the start and then retarded; the second is at first slow, then gradually more rapid. Nitrate is used up in the forest soil as soon as formed; in beaker studies in the laboratory it accumulates only from layer 3.

Hesselman divided all forest soils into nitrifying and non-nitrifying, the former comprising the birch, oak and other soils, in which the organic matter is well intermixed with the mineral materials, while the latter comprise the evergreen soils rich in mosses and lichens, in which the organic matter is largely "raw humus" and "dry peat;" the first are brown soils, the second podsol soils. Melin<sup>4</sup> has shown that an active liberation of nitrogen, especially in the nitrate form, or a factor closely connected with it, favors an optimum development of mycorrhiza and is of great importance in establishing the equilibrium between the two symbionts. Nitrates are produced in forest soil even at pH of 3.9-4.1. In soil layers exposed to light,<sup>4</sup> nitrification takes place actively;

<sup>4</sup> Melin, E. Meddel. Stat. Skogsförsöks., H. 23, No. 6-7. 1927; Wittich, W. Diss. Forstl. Hochsch. Eberswalde. 1926.



in shady forests, the organic nitrogen is changed to ammonia under thin cover, but little nitrogen is liberated under the thick cover of "raw humus." The nature of forest vegetation and tree growth depend largely upon the transformation of the nitrogen in the soil, and in their turn affect the nitrogen conditions.

Aaltonen<sup>5</sup> expressed his doubts whether nitrification plays such a predominant rôle in the regeneration of forests. His results point to the fact that large quantities of ammonia are formed in forest soils and that trees growing in ordinary moss forests do not receive the nitrogen in the form of nitrate. He agreed, however, with Hesselman that forest soils can be classified into nitrifying (grass-herb forests) and non-nitrifying (moss forests). The better the forest type the higher is the nitrogen content of the soil and the larger is the amount of nitrogen in a mineralized form (ammonia and nitrate). According to Nemec,<sup>6</sup> the resin content of the organic matter and the acidity of the soil are important factors, both in the formation of humus in the surface layer of forest soils and in the rate of nitrification, these processes varying inversely with the amount of resins present and the degree of acidity.

*Microflora of forest soils.* The microflora of forest soils consists of a large number of fungi and numerous bacteria; among the former the Basidiomycetes play not the least important rôle. The earlier students of forest soils (Müller, Ramann) recognized this fact and were even inclined to consider the fungi as the only agents in the microbial transformations in those soils. The relation between the fungi and bacteria in forest soil can well be brought out by the determination of their relative abundance by the plate method; the fungus counts obtained by this procedure represent, however, only a very low minimum. In normal field and garden soils bacteria predominate over fungi, both in total numbers and in the variety of activities, while in forest soils the fungi are much more abundant, especially when the actual amount of microbial cell substance is computed. Ramann<sup>7</sup> reported that the numbers of bacteria per gram of organic matter in various forest soils range from 2,050,000 to 59,880,000 and the numbers of filamentous fungi from 66,000 to 10,280,000 per gram (and even more). This number of fungi represents only the spores found in a given quantity of soil. The plate method gives no information at all concerning the great

<sup>5</sup> Aaltonen, V. T. Inst. Qnaest. Forest. Finland. Ed. 10, Helsinki. 1926.

<sup>6</sup> Nemec, A. Compt. Rend. Acad. Sci. (Paris), 185: 1145-1155. 1927.

<sup>7</sup> Ramann, E., Remele, C., Schelhorn and Krause, M. Ztschr. Forst. Jagdwes. 31: 575-606. 1899.

mass of fungus mycelium, especially of the Basidiomycetes, which permeates all the organic matter in forest soils. Students were, therefore, warned not to rely upon the plate count of fungi. It was suggested that the abundance of mycelium should be determined by making a microscopic examination of the soil, which reveals the organic matter to consist of a mass of hyphae holding together some of the plant residues. As shown above, Falck even attempted to differentiate various types of forest soil on the basis of the active microflora and microfauna and the processes of transformation of organic matter brought about by these organisms; the nature of the microbial population of forest soils depends of course upon the plant residues, the soil and the environmental conditions. Certainly so far as the initial stages of decomposition of organic matter in forest soils are concerned, the fungi were found to be by far more active than the bacteria.

Koning<sup>8</sup> found a specific fungus flora on the leaves and needles fallen to the ground and undergoing decomposition. These fungi were much the same as those found in the air above. They varied considerably with the season of year. In May and June the following fungi predominated: *Pen. glaucum*, *Monilia humicola*, *Hormodendrum pallidum*, *Orthobotrys superba*, *Mortierella pusilla*, *Pen. cinereum*, *Botrytis vulgaris*, *Monosporium viridescens*, *Tilachlidium humicola*, *Stemphylium botryosum* and 2 species of yeasts. In July, *Sporotrichum foliicola* also developed in abundance. In August, other species appeared, including the genera *Eurotium* and *Gymnoascus*. In September, the following forms developed, in addition to those found during May to July: *Stemphylium piriforme*, *St. botryosum*, *Alternaria termis*, *Trichoderma koningi*. The mite *Tyroglyphus longior* was found to feed on yeasts and the mycelium and spores of fungi. On the other hand, *Cephalosporium koningi* was not found in the air but only in the soil. A number of other species of fungi were found in the air and on the leaves and needles. One of the most common and abundant organisms is *Trichoderma koningi*, which appears largely in September and in October upon the dying or dead leaves of *Quercus*.

These and many other studies of the fungus population occurring on the plant residues in forest soils established the fact that numerous

<sup>8</sup> Koning, C. J. Arch. Neerl. Sci. Ex. Nat. II, 9: 35-107. 1904. More recent information concerning the fungus flora of forest soils is given by Samoutsevitch, M. M. Materials of Mycol. Phytopath. Leningrad 6: 204-213. 1927; Dogiel, V. and Effremoff, G. Arb. Leningrad Naturwiss. Gesell., 55: 1927; Pistor, R. Centrbl. Bakt. II, 80: 169-200, 378-410. 1930.

Myxomycetes, Ascomycetes, Fungi Imperfecti and Basidiomycetes, and, to a less extent, Phycomycetes are found<sup>9</sup> on decomposing wood. Although there is no doubt that these fungi take an active part in various decomposition processes, it is difficult to make any definite statement concerning the rôle of certain specific organisms, before more experimental evidence has been obtained.

The bacterial population of forest soils has been reported<sup>10</sup> to consist of smaller numbers of organisms than in the case of field and garden soils, especially soils receiving stable manures. A direct relation was found to exist between the bacterial content of the soil and the evolution of CO<sub>2</sub>. The reaction of the soil, as expressed by the pH, was found to control the number of bacteria, as determined by the plate method; with a constant pH, the numbers increase with an increase in the content of organic matter and air capacity. It is important to note that nitrogen-fixing bacteria in forest soils can withstand a higher acidity than in agricultural soils. When different soils are compared, it is found that soils of mixed forests contain more bacteria than soils of deciduous forests, and these more than soils of coniferous forests. One would expect, from a knowledge of the influence of acidity upon bacterial development, that soils of forests of mixed coniferous and deciduous trees, which are generally more acid than soils of deciduous forests, would contain fewer bacteria. Various anaerobic forms, especially the butyric acid bacteria, are found abundantly in forest soils, while the number of nitrifying bacteria is very low. Romell<sup>11</sup> found that these bacteria are present in forest soils in the form of zoogaea; in this stage they can survive in soil for a long time even under unfavorable conditions.

Both aerobic and anaerobic cellulose decomposing bacteria are found in forest soils even at a pH of 4.3. The large number of anaerobic bacteria in forest soils (from 500,000 to 5,000,000 per gram of soil) should attract particular attention; the same is true of the anaerobic nitrogen fixing organisms (100 to 10,000 per gram). Aerobic nitrogen fixing bacteria are either entirely lacking in forest soils or are present

<sup>9</sup> Kauffman, C. H. *Mich. Acad. Arts Lett.*, **9**: 169-218. 1928; Povah, A. H. *Ibid.*, 253-272; Hagem, O. 1907 (p. 221); Süchting, H. *Forstl. Wochschr. Silva*, **17**: 321, 329. 1929.

<sup>10</sup> Bokor, R. *Math. es Term. Ertesito (Budapest)*, **43**: 561-572. 1926; *Biochem. Ztschr.*, **181**: 302-304. 1927; Fehér, D. *Biochem. Ztschr.*, **206**: 416-435. 1929; *Acta Forens. Fenn.*, **34**. 1929.

<sup>11</sup> Romell, L. G. *Meddel. Stat. Skogsförsöks.*, **24**: 57-66. 1928.

there only to a very limited extent; this points definitely to the rôle of the anaerobic bacteria in the fixation of nitrogen in these soils.<sup>12</sup>

*Microfauna of forest soils.* The importance of the animal population in forest soil has been recognized as a result of the work of Darwin, Hensen, Müller, Wollny, and others. P. E. Müller has shown in 1878 that the formation of a good mull soil depends upon the presence of earthworms. The methods used at that time could not enable one to distinguish the small species of earthworms living in raw-humus soils, hence the idea was advanced that the latter are devoid of this group of organisms. Testaceous rhizopods, however, were found to be numerous in raw-humus and scarce in mull soils, as shown by Müller. Bornebusch<sup>13</sup> has made recently an extensive study of the microfauna of forest soils and demonstrated that the difference in the animal population is rather quantitative in nature, in contradistinction to that of the higher flora which is more qualitative.

The fauna of a deciduous forest mull soil was found to consist chiefly of earthworms (50–80 per cent of the total weight of the fauna), which mix the plant residues with the inorganic soil particles. Among these, the two most common groups are the turgid worm (*Allolobophora turgida*) and the common earthworm (*Lumbricus terrestris*). Among the arthropods, the millipeds, *Trichoniscus* and certain ground-beetle species are most common. The slower the decomposition of the organic matter in the soil, the less is the total weight of the animals and the greater is the number of arthropods, click-beetle larvae, diptera larvae, collembola and mites, which increase also in proportion as the earthworms grow fewer. A spruce forest mull fauna comprises earthworms only to the extent of half the weight of the total fauna. Here belong *Lumbricus rubellus*, *L. castaneus* and *Dendrobaena* species. A deciduous forest raw humus fauna contains only from a few to about 20 per cent of the weight of the animals as earthworms (largely *Dendrobaena*), which inhabit the layer of organic matter, hardly mixed with the mineral soil. The fauna consists largely of arthropods, among which diptera larvae, click-beetle larvae, millipeds, collembola and mites predominate. A spruce forest raw humus fauna contains few earthworms, not more than 10 per cent of the total weight of the fauna, largely confined to *Dendrobaena octoedra*. The fauna is rich in arthropods, *Trichoniscus* being absent. Millipeds are rare,

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<sup>12</sup> Dügge, M. Schweiz. Ztschr. Forstwes., 1923, 1–37; Beibl. Vierteljahr. Naturf. Gesell. Zürich, 73: 307. 1928; Fehér, D. Arch. Mikrob., 1: 464–492. 1930; see also p. 106.

<sup>13</sup> Bornebusch, C. H. The fauna of forest soil. Copenhagen. 1930; Trägarth, I. Särt. Skogshögsk. Festskr. 1928, p. 795–813; see also Ramann, E. Intern. Mitt. Bodenk., 1: 138–164. 1911; Blake, I. M. Illinois Biol. Monogr., 9: 7–93. 1924; 10: 7–117. 1924; Herold, W. Ztschr. Morphol. Ökol. Tiere, 4: 398. 1925; Pillai. Ztschr. angew. Entom., 8: 1–30. 1922; Soudek, S. Bul. Ecol. Agron. Brno. 1928.



while mites are in abundance and far exceed the colembolla. Diptera larvae and especially click-beetle larvae predominate in weight. Geophilidae are very abundant.

As a measure of activity of the fauna, the weight of the animals was found by Bornebusch to be a more reliable index than the number. The respiration of the animals contributes a large part to the total respiration of forest soils. In mull soils, where decomposition is very active, the weight of the animals preponderates but their number is small. When decomposition is slow, a larger number of animals is found, the latter being, however, much smaller in size (table 89).

The deep and friable mull soil in deciduous forests is believed to be a direct result of the activity of the larger earthworms (*L. terrestris*, *L. rubellus*, *Allolobophora turgida*, *A. trapezoides*, *Eisenia rosea*, etc.). Arthropods are of the greatest importance in raw-humus soils, where the organic matter is deposited on the surface and is not intermixed with the mineral soil. Bornebusch concluded that the fauna is very active in the processes of decomposition in forest soil, especially the large earthworm species. Deciduous trees, especially those bearing easily decomposable leaves, a flora of herbaceous plants and good shelter will favor their development; coniferous trees are frequently injurious to most species of earthworms.

The fauna of the forest soil comprises animals that either spend their whole life or the larger part of it in the soil, obtaining their sustenance from the living and dead organic materials, and animals that spend only a certain part of their life cycle in the soil, either as pupas and hibernating larvae or as full-grown insects, and do not consume any food in the soil. The latter group makes up only a small part of the fauna. Bornebusch divides the soil fauna into three groups:

1. Animals that live on deciduous organic matter in the soil and mix the same with the mineral part of the soil. Only earthworms (*Lumbricidae*) belong to this group.
2. Animals which digest and decompose deciduous organic matter, without mixing it with the mineral top soil. Here belong *Gastropoda* (snails), *Enchytraeidae* (potworms), *Isopoda* (woodlice), *Diplopoda* (millipedes), *Acarina* (mites), *Collembola* (springtails), *Diptera* (two-winged insects), *Elateridae* (click-beetles), and other insects, except *Staphylinidae* and *Carabidae*.
3. Predaceous animals, feeding on other animals thus keeping them in check. Here belong *Chilopoda* (centipedes), *Arachnida* (except *Acarina*), *Staphylinidae* and *Carabidae*.

The number of protozoa in forest soils varies from 2,500 to 10,000 per gram; they are mostly in a cyst condition, with considerable variation at different periods of the year and in different forest soils. No definite

relation seems to exist between the bacteria and the protozoa in these soils.<sup>14</sup>

*Decomposition of organic matter in forest soils.* The major problem of the fertility of forest soils is the rapidity and nature of decomposition of the tree residues that drop yearly to the ground from the trees, shrubs and mosses making up the forest cover. Ebermeyer<sup>15</sup> reported in 1876 that the average annual fall of tree residues ranges from 1,673 to 4,806 pounds of dry litter per acre, with an average of 2,732 pounds. There was considerable variation from year to year, depending also on the fertility of soil, nature of vegetation and climate. The nitrogen content of the litter varied from 0.8 to 1.0 per cent. Alway and Zon found that the forest floor, or surface organic layer, contains, on an average, 28.142 pounds of material (16.553–33.987 pounds), with 28.05 per cent ash, 0.98 per cent nitrogen, 0.86 per cent CaO, 0.17 per cent P<sub>2</sub>O<sub>5</sub>, 0.15 per cent K<sub>2</sub>O and 0.22 per cent SO<sub>3</sub>. The yearly fall of pine litter per acre was calculated as 1,798 to 2,464 pounds, with the following concentration, in pounds, of the important minerals: 41.6–105.0 ash, 8.4–17.7 nitrogen, 8.6–21.3 CaO, 2.2–3.5 P<sub>2</sub>O<sub>5</sub>, 2.4–4.3 K<sub>2</sub>O and 2.3–6.7 SO<sub>3</sub>.

In order that these nutrient elements be again made available for plant growth, the organic residues have to be first decomposed by micro-organisms. It is commonly assumed that the disintegration of tree residues consists in the chemical simplification of the various constituents from a greater to a lesser complexity, finally leading to their transformation into carbon dioxide, ammonia, water, and, under anaerobic conditions, also into hydrogen and methane. The assumption that various intermediary substances are formed in this process has never been sufficiently substantiated. There is no doubt that when celluloses<sup>16</sup> are decomposed by anaerobic bacteria large quantities of organic acids and alcohols are produced, but these should be considered as final products of anaerobic metabolism rather than as intermediary products; these acids and alcohols are further decomposed as soon as conditions are made aerobic and the reaction is favorable.

There is no justification for speaking of "simplification" of complex plant constituents when these plants are decomposed by fungi. There

<sup>14</sup> Fehér, D. and Varga, L. Centrbl. Bakt. II, 77: 524–542. 1929.

<sup>15</sup> Ebermeyer, E. Die gesammte Lehre der Waldstreu. Berlin, 1876; see also Ramann, E. Die Waldstreu und ihre Bedeutung für Boden und Wald. Berlin, 1893; Forstliche Bodenkunde und Standortslehre. Berlin, 1893; Henry, E. Les sols forestiers. Paris, 1908; Möller, A. Der Waldbau. J. Springer, Berlin. 1929; Alway, F. J. and Zon, R. Jour. Forestry, 28: 715–727. 1930.

is more sound evidence that the chemical processes involved in the decomposition of tree products by microorganisms consist merely in the complete destruction of the more readily available substances, followed by a slow but gradual disappearance of those that are less readily decomposed: the more resistant plant constituents and the substances synthesized by microorganisms contribute to the formation of the residual material.

The chemistry of wood and wood products still represents a series of complicated problems, and, unless these are first carefully worked out, one could not expect to unravel the chemistry of their decomposition, as well as the chemistry of the residual soil humus. It is sufficient to call attention to the lignin and cellulose in the wood and litter,

TABLE 90

*Analyses of sound and decomposed Douglas Fir Wood (Rose and Lisse)*

PLANT CONSTITUENTS	PER CENT OF DRY MATERIAL		
	Sound wood	Partially rotted wood	Completely rotted wood
Cold water-soluble.....	4.03	1.75	1.16
Hot water-soluble.....	2.23	4.19	7.77
Alkali-soluble.....	10.61	38.10	65.31
Cellulose.....	58.96	41.66	8.47
Pentosan.....	7.16	6.79	2.96
Methyl pentosan.....	2.64	3.56	6.06
Methoxyl group.....	3.94	5.16	7.80
Ether extract.....	2.71	2.05	2.72
Ash.....	0.15	0.15	0.65

which make up, in the form of ligno-celluloses, 60 to 70 per cent of the total organic matter. Due to differences in the methods used by different investigators for the determination of these constituents, both in the sound wood and in decomposed material, the results obtained are frequently very conflicting; it is only within recent years that certain positive information has been obtained concerning the processes involved in the decomposition of wood and tree products.

Hartig<sup>16</sup> was the first to demonstrate in 1878 that the rotting of wood can be brought about in two different ways: 1. certain fungi cause the disappearance of the cellulose (in the so-called "brown rot") and the accumulation of the lignin, thus resulting in a product richer in carbon

<sup>16</sup> Hartig, R. Zersetzungserscheinungen des Holzes. Berlin. 1878.



and in alkali-soluble substances; 2. other fungi decompose the lignin (in the "white rot") leaving a residue consisting largely of cellulose.

Rose and Lisse<sup>17</sup> found (table 90) that when wood is rotted by fungi there is a gradual decrease in the relative concentration of cellulose and pentosan and an increase in lignin, as determined by the methoxyl content and the amount of alkali-soluble materials. The nature and rapidity of decomposition of the wood depend upon the nature of the infecting organism. Certain Hymenomycetes causing "brown rot" of wood brought about a loss of 10 to 50 per cent of the total material in 6 to 10 months.<sup>18</sup> The cellulose was reduced from 60 to 6 per cent

TABLE 91

*Chemical composition of decomposed wood, as compared with sound wood*  
On per cent basis of dry material

CHEMICAL CONSTITUENTS	SOUND WOOD		ROTTED WOOD		WOOD FROM PEAT	FOSSILIZED WOOD (OAK)
	Chest-nut	Cypress	Decomposed wood*	Decomposed wood†		
Ether-soluble.....	2.66	3.53	1.48	6.91	1.54	0.84
Cold and hot water-soluble.....	7.08	3.18	1.26	5.61	0.87	0.56
Alcohol-soluble.....	3.27	1.92	5.05	3.30	1.34	1.82
Hemicelluloses.....	15.23	11.16	4.72	14.43	8.15	3.79
Cellulose.....	32.58	37.62	2.16	27.78	6.12	3.38
Lignin‡.....	22.05	28.21	71.14	23.61	65.02	70.74
Protein.....	0.54	0.66	1.31	2.18	5.37	2.21
Ash.....	0.54	0.76	0.65	2.25	3.85	4.40
Total accounted for.....	83.95	87.04	87.77	86.07	92.25	87.74

\* Wood which has undergone the so-called process of "destruction."

† Wood which has undergone the so-called process of "corrosion."

‡ Free from ash and nitrogen.

in one year, while the loss in the lignin was only 3 per cent; this small loss could be all accounted for by a reduction in the methoxyl content (2.8 per cent). However, although the lignin did not decompose as rapidly as the cellulose, hemicelluloses and proteins, it still underwent some decomposition, slow to be sure, under aerobic conditions, as shown by an increase in alkali solubility.

<sup>17</sup> Rose, R. E. and Lisse, W. M. Jour. Ind. Engin. Chem., 9: 284-287. 1917;  
Bray, M. W. and Andrews, T. M. Jour. Ind. Engin. Chem., 16: 137-139. 1924.

<sup>18</sup> Waksman, S. A. and Stevens, K. R. Jour. Amer. Chem. Soc., 51: 1187-1196. 1929.

Under aerobic conditions, wood and tree residues are decomposed largely through the agency of fungi; there may result either a rapid disintegration of the cellulose with the accumulation of lignin or a gradual decomposition of both cellulose and lignin, and frequently even an accumulation of cellulose, as shown previously (p. 401).

A comparison of the chemical composition of sound and decomposed wood is given in table 91,<sup>18</sup> which brings out the chemical changes that have taken place as a result of the decomposition of the various constituents of the wood by different groups of microorganisms. According to Grüss,<sup>19</sup> in the decomposition of wood by fungi, the lignins are changed into lignic acid. Wehmer<sup>20</sup> found that the fungus *Merulius lacrymans* brings about the decomposition of the cellulose in wood and the transformation of the lignin into "humins;" as a result of this there is a considerable increase in titratable acidity. Although no organic acids could be demonstrated, it was believed that the formation of water- and alkali-soluble "humic acids" and "humins" was responsible for this phenomenon. The carbon content of cellulose is 44.2 per cent, of sound wood 51.5 per cent and of lignin 62 to 64 per cent. The carbon content of the decomposed wood was 56.8 per cent, of the water-soluble material 46.4-51.6 per cent, of the alkali soluble portion precipitated by hydrochloric acid 64 per cent, and of the insoluble residue 60.5 per cent. When one hundred parts of wood were decomposed by the fungus, 50 parts were liberated in the form of carbon dioxide and water; the residual 50 parts consisted of 15 per cent water-soluble material, 35 per cent alkali-soluble and 50 per cent insoluble material.

According to Kürschner,<sup>21</sup> *Mer. lacrymans* and other wood-destroying fungi attack largely the cellulose in the wood, while the lignin is transformed into a complex mixture of changed and unchanged depolymerized substances of a humic-like nature. Strache<sup>22</sup> analyzed the inner portion of a 1000 year old pine and found that only traces of cellulose were left, while the lignin has been changed, in the absence of oxygen, into "humic acid," which was thus considered to be the first step in the formation of lignite and brown coal. The residue of the action of *Mer. lacrymans* upon pine wood was found by Schwalbe<sup>23</sup> to consist of 73 per

<sup>18</sup> Grüss, J. Ber. deut. bot. Gesell., **41**: 48-52, 53-58. 1923.

<sup>20</sup> Wehmer, C. Brennstoff-Chem., **6**: 101-106. 1925; Ber. deut. chem. Gesell. **48**: 130-134. 1930.

<sup>21</sup> Kürschner, K. Ztschr. angew. Chem., **40**: 224-232. 1927; see Barton-Wright, E. C. and Boswell, J. G. Biochem. Jour., **23**: 110. 1929.

<sup>22</sup> Strache, H. Brennstoff-Chem., **8**: 21-22. 1927.

<sup>23</sup> Schwalbe, C. G. and Ekenstam, A. Cellulosechem., **8**: 13-15. 1927.

cent lignin, 15 per cent cellulose, 8 per cent other carbohydrates and 4 per cent resins; the fats and pentosans were all destroyed; 64.2 per cent of the lignin was soluble in 5 per cent NaOH solution and contained less methoxyl (10.2 per cent) than lignin of fresh wood. Brandl<sup>24</sup> recorded the presence of 24.9 per cent acid hydrolyzable material, in-

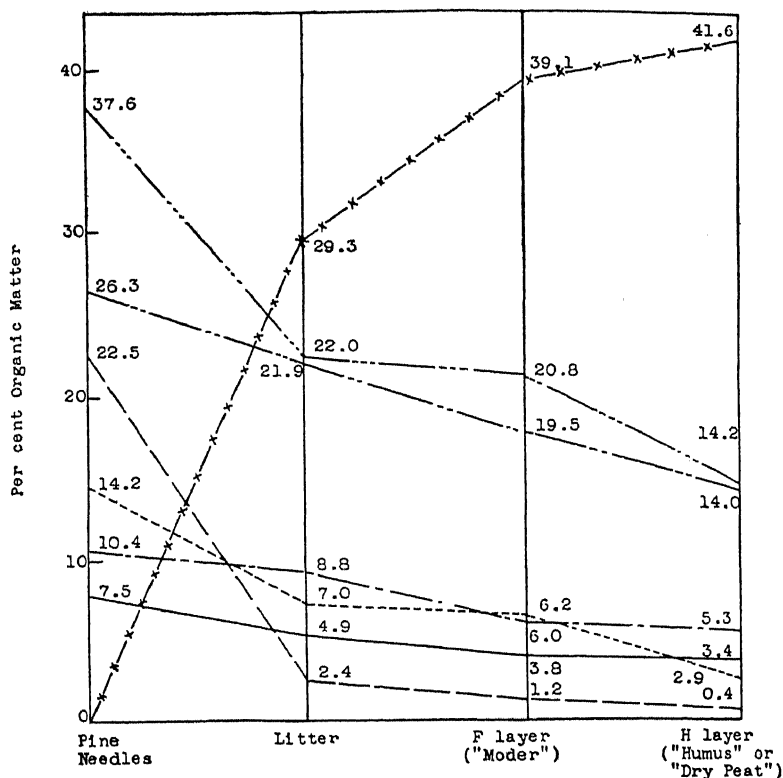


FIG. 60. Decomposition of the various constituents of plant residues in the transformation of organic matter in forest soil. Ether soluble material — · — · —, alcohol soluble material — — —, water soluble material — — —, pentosans — · — · —, cellulose — — —, lignin — — —, humus — x — (from Groszkopf).

cluding 8.1 per cent cellulose, and 75.1 per cent non-hydrolyzable material in decomposed oakwood; the latter fraction was separated into lignin

<sup>24</sup> Brandl, A. Brennstoff-Chem., 9: 89-94. 1928; Falck, R. and Coordt, W. Ber., 61: 2101-2106. 1928; Campbell, W. G. and Booth, J. Biochem. Jour., 23: 566-572. 1929; Kleberg, T. Diss. Münster, 1927.

(36.35 per cent) and humic acid (38.75 per cent), on the basis of solubility in sodium bicarbonate solution. As a result of digestion of wood by insects, there was also found a gradual increase in the lignin content and a decrease in the cellulose content.<sup>25</sup>

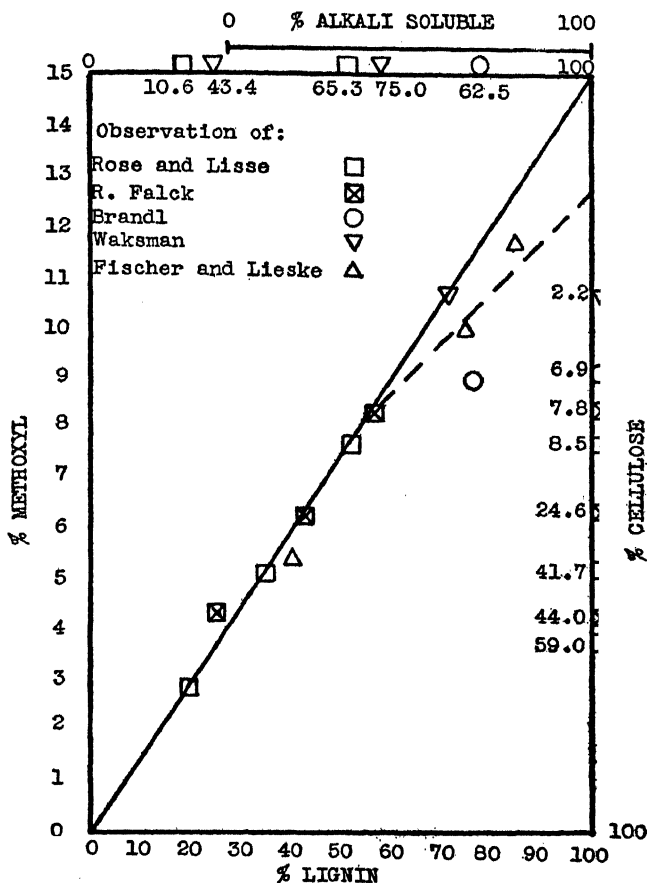


FIG. 61. Lignin and cellulose transformation in the decomposition of wood by microorganisms (from Fuchs).

Fig. 60<sup>26</sup> illustrates the gradual transformation of the various chemical constituents of tree residues in the process of their decomposition. The forest material is divided, according to Hesselman's system, into the

<sup>25</sup> Fischer, F. and Lieske, R. *Biochem. Ztschr.*, **203**: 351-362. 1928.

<sup>26</sup> Groszkopf, W. *Brennstoff-Chem.*, **7**: 293-299. 1926; **10**: No. 9, 11, 1929.

upper layer or litter, the second or F-layer, and the lowest or H-layer, the last being just above the mineral soil and in which the plant residues can no longer be recognized. According to Groszkopf, lignin decomposing organisms cannot withstand a high acidity or exclusion of air; under these conditions, both cellulose and lignin are changed into transformation products of an insufficient degree of oxidation and decomposition. The cellulose is transformed into black "humoids" and the lignin, through various intermediary products, into "humic acids," or complex phenol-carbonic acids or their anhydrides, which can be further decomposed by bacteria only with great difficulty.

TABLE 92

*Proximate chemical composition of plant products and of forest "humus"*  
On per cent basis of dry materials

NATURE OF PLANT MATERIAL	COMPOSITION OF THE F- AND H-LAYERS OF THREE TYPICAL FOREST SOILS						PINE NEEDLES	OAK LEAVES	HYPNUM MOSS
	A(F)	A(H)	B(F)	B(H)	C(F)	C(H)			
Reaction, pH.....	5.6	4.9	5.1	4.4	4.4	4.3			
Cold and hot water soluble fraction.....	5.80	2.73	5.03	4.66	5.14	3.63	13.02	15.32	8.41
Hemicelluloses.....	15.28	12.39	15.48	17.87	17.50	17.30	14.68	15.60	18.92
Cellulose.....	9.44	2.56	7.28	3.84	9.62	5.64	18.26	17.18	24.75
Lignin.....	39.30	50.39	38.38	37.29	42.26	44.88	27.63	29.66	21.13
Crude protein.....	8.29	7.51	8.02	7.04	6.84	5.15	8.53	3.47	4.16
Ether-soluble fraction.....	4.41	2.99	5.21	3.96	3.58	3.94	7.65	4.01	4.58
Ash.....	9.20	11.61	7.94	13.67	6.05	10.57	3.08	4.68	4.33
Total.....	91.72	90.18	87.34	88.33	90.99	91.11	92.85	89.92	86.28

Fuchs<sup>27</sup> summarized the relations between the lignin and cellulose content and the amount of alkali-soluble material in wood, which has undergone decomposition under various conditions, as shown in fig. 61.

An analysis of the F and H layers of three typical forest soils, as compared with fresh tree products is given in table 92. A—represents a Northern hardwood-spruce forest, B—a forest of mixed coniferous and deciduous trees, C—a spruce forest with a heavy growth of hypnum moss.<sup>28</sup>

<sup>27</sup> Fuchs, W. Brennstoff-Chem., 11: 108. 1930.

<sup>28</sup> Waksman, S. A., Tenney, F. G. and Stevens, K. R. Ecology, 9: 126-144. 1928.

Nemec<sup>29</sup> recorded the presence of 7.10 to 13.13 per cent pentosan in the upper layer of forest soil, as compared with 8.72 to 14.06 per cent in the dead needles and leaves fallen to the ground. The pentosan content diminished rapidly with depth. The benzol-alcohol extract of the surface layer of soil was 5.30 to 12.68 per cent, as compared with 12.19 to 25.47 per cent in the corresponding dead tree residues. He found that the lower this fraction in the soil, the greater is the amount of nitrate produced.

A definite relation was reported<sup>29</sup> to exist between the abundance of organic matter and the acidity of coniferous forest soils; the greater the organic matter content, the lower is the pH value. In the case of deciduous and mixed forests, no such relation was observed. The

TABLE 93

*Influence of reaction upon the nitrogen content and nitrogen liberation in forest soil*

FOREST TYPE	REACTION	NITROGEN CONTENT OF ORGANIC MATTER	MINERALIZED NITROGEN, PER CENT OF TOTAL	
			Original sample	After 2 months incubation
	pH	per cent		
<i>Cladina</i> .....	3.6			
<i>Calluna</i> .....	4.2	1.495	0.220	1.074
<i>Vaccinium</i> .....	4.6	1.666	0.335	1.207
<i>Myrtillus</i> .....	4.8	1.796	0.383	1.819
<i>Oxalis-Myrtillus</i> .....	5.2	2.234	0.484	2.868
<i>Oxalis-Majanthenum</i> .....	5.0	2.795	0.551	4.425

higher the acidity of the "humus" layers the lower is the nitrogen content of the organic matter, a phenomenon found to hold true of all forests. This is brought out<sup>30</sup> in table 93. The degree of decomposition of the organic matter in soil shows<sup>31</sup> also a very good correlation with the acidity and mobilization of nitrogen in various forest types, according to Cajander's classification.<sup>32</sup> The growth of mycorrhiza fungi is closely connected with the reaction, as illustrated<sup>33</sup> in fig. 62, a fairly high acidity being required for the optimum development of two typical fungi.

<sup>29</sup> Nemec, A. Forstwirtschaft. Centrbl., 73: 90-105, 117-131, 178-187. 1929.

<sup>30</sup> Aaltonen, V. T. Comm. Inst. Quest. Forest. Finland., 10, 1926; see also Nemec, A. and Kvapil, K. Ztschr. Forst. Jagdw., 58: H 8-9. 1926; Clarke, G. R. Oxford Fores. Mem., 2, 1924.

<sup>31</sup> Nemec, A. Ztschr. Forst. Jagdwes., 60: 385-424, 471-513. 1928.

<sup>32</sup> Cajander, A. K. Acta forest. fennica., 31. 1926.

<sup>33</sup> Melin, E. Bot. Notiser, 1: 38. 1924.

Weis<sup>34</sup> found that the nitrogen content of the organic matter increases with increasing depth of forest soils, as shown in the following summary:

NATURE OF LAYER	RAW HUMUS + LEACHED SAND	HARDFAN	LAYER UNDER HARDFAN	SUBSOIL
Horizon.....	A <sub>1</sub> - A <sub>2</sub>	A <sub>3</sub> - B <sub>1</sub>	B <sub>2</sub>	C
Per cent of nitrogen in organic matter.....	2.08	2.24	3.99	8.44

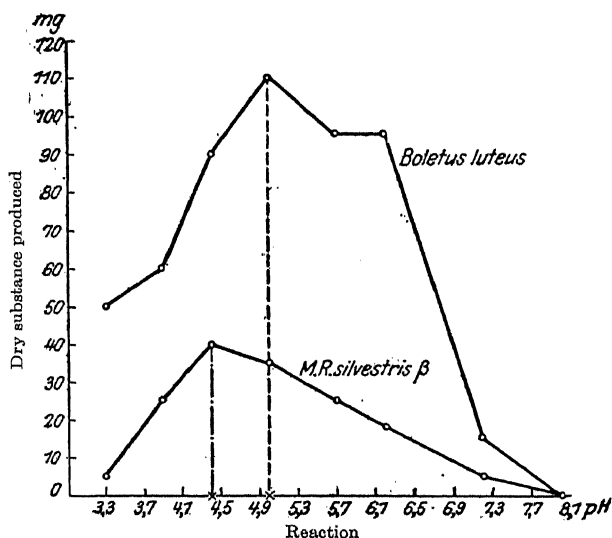


FIG. 62. Influence of reaction upon the growth of two typical mycorrhiza fungi (Melin).

In some instances as much as 13 to 16 per cent and even 22.2 per cent nitrogen has been found in the organic matter of the deepest layers of the profile. In order to explain this exceptionally high nitrogen content, it has been suggested that the organic matter in the lower depths of soil is true colloidal material, which is highly nitrogenous in nature and is capable of being washed down most rapidly.

*Formation of ammonia and nitrate-nitrogen in forest soils.* The liberation of nitrogen in an available form takes place more rapidly in the

<sup>34</sup> Weis, Fr. Kgl. Danske Vidensk. Selsk. Biol. Meddel., 7: H. 9. 1929.

decomposing (F-layer) horizon than in the humus (H-layer) horizon. According to Hesselman,<sup>35</sup> the nitrogen left in the humus layer is more firmly bound and becomes more resistant to decomposition. Süchting<sup>36</sup> found that the nitrogen complexes of "raw-humus" consist largely of substances which undergo decomposition with great difficulty, as pyridin, chinolin and acridin. Melin<sup>37</sup> demonstrated that the activities of mycorrhiza fungi are less in the humus than in the decomposing horizon.

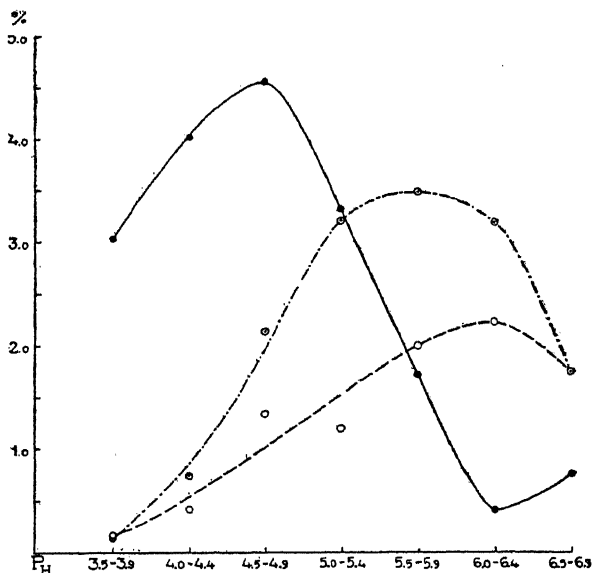


FIG. 63. Relation between soil reaction and the transformation of organic nitrogen into ammonia and nitrate nitrogen in forest soil: —•— ammonia-nitrogen, per cent of total nitrogen; ○ nitrate nitrogen, per cent of total; ○ nitrate nitrogen, produced in soil on incubation, per cent of total (from Hesselman).

When the tree residues and other forest vegetation fall to the ground, they begin to undergo rapid decomposition, with very little nitrogen becoming liberated as nitrate. Only after the carbon-nitrogen ratio has been made narrower and the cellulose and hemicelluloses have diminished markedly, does nitrate begin to accumulate. Nitrate forma-

<sup>35</sup> Hesselman, 1926 (p. 6).

<sup>36</sup> Süchting, H. Forstwirtschaft. Centrbl., 47. 1925.

<sup>37</sup> Melin, E. Medd. Statens Skögsförsöks. H. 23. 1927.



tion usually accompanies the latter stages of decomposition, after the growth of the numerous fungi is followed by the development of the animal and bacterial populations.

The earlier students of forest soils believed that no nitrates are formed in these soils at all. Migula,<sup>38</sup> however, established in 1900 the fact that not only is this assumption unjustified, but that nitrifying bacteria can actually be demonstrated in forest soils. Weis<sup>39</sup> established the occurrence of nitrates in natural forest soils; under favorable conditions, a sufficient amount of nitrogen required for plant nutrition is thus produced. The mull-types of soil offer conditions very favorable to nitrate formation. The season of year has an important influence, the lowest amounts of nitrate being found in the summer months, due to its rapid absorption by plants. The nature of the soil and of the forest vegetation are also of considerable importance in this connection.<sup>40</sup> Fig. 63 brings out the relation between the reaction of forest soil and the formation of nitrates. Table 94 shows the influence of forest soil material upon the formation of ammonia and nitrate nitrogen, following the decomposition of the organic matter under favorable conditions.

Soils of mixed forests show a more active formation of nitrate than soils of pure coniferous or deciduous forests.<sup>41</sup> The formation of nitrate in forest soil depends upon a larger number of factors than in the case of field and garden soils. They include not only the question of reaction, aeration, moisture supply, light supply, soil type and depth of soil, but also the nature of surface vegetation, age of forest, nature of the organic matter layer and extent of its decomposition, abundance of inorganic matter in the organic layers, etc.

The presence of tannins, resins and terpenes in the forest products has an injurious effect upon nitrate formation.<sup>42</sup> This may be partly the reason for the low nitrate formation in evergreen soils. It is interesting to note that evergreen trees prefer the nitrogen as ammonia while deciduous trees prefer nitrate nitrogen.<sup>43</sup> Mattern<sup>44</sup> also reported that the slow rate of nitrification and nitrogen-fixation in a beech forest

<sup>38</sup> Migula, W. *Centrbl. Bakt.* II, 6: 365-370. 1900.

<sup>39</sup> Weis, F. *Centrbl. Bakt.* II, 28: 434-460. 1910; Müller, P. E. and Weis, F. *Naturw. Ztschr. Land. Forstw.*, 5: 52. 1907.

<sup>40</sup> Vogel and Falckenstein. *Intern. Mitt. Bodenk.*, 3: 494. 1913.

<sup>41</sup> Nemec, A. and Kvapil, K. *Ztschr. Jagd. Forstw.*, 59: 321-352, 385-412. 1917; see also Fehér, D. *Arch. Mikrob.*, 1: 381-417. 1930.

<sup>42</sup> Koch, A. and Oelsner, A. *Centrbl. Bakt.* II, 45: 107-117. 1916.

<sup>43</sup> Vater, H. *Tharandt. Forstl. Jahrb.*, 59: 261. 1909.

<sup>44</sup> Mattern, M. *Bot. Arch.*, 22: 1-132. 1928.

*Nitrogen availability in the decomposition of organic matter in different forest soil horizons (Bornebusch)*

On basis of dry material

DESCRIPTION OF SOIL	DEPTH OF SOIL	pH	TOTAL ORGANIC MATTER	TOTAL NITROGEN	AFTER 6 WEEKS INCUBATION OF 1 KG.M. OF MATERIAL	
					NH <sub>4</sub> -N	NO <sub>3</sub> -N
	cm.		per cent	per cent	mgm.	mgm.
<i>Beech-mull:</i>						
Newly-fallen leaves.....	1.5	5.9	78	1.15	Trace	2.2
Old leaf layer.....	0.5	6.1	54	0.92	84	1,200.0
Worm casts.....		5.8	22	0.42	8	264.0
Upper mull soil.....	0-5	5.4	14	0.29	4	48.0
Lower mull soil.....	5-15	5.2	7	0.15	2	7.5
Topsoil.....	35	4.6	4	0.08	0	0.5
Subsoil.....	70	5.1				
Subsoil.....	90	5.6				
Subsoil.....	200	7.5				
<i>Beech-raw humus:</i>						
Newly-fallen leaves.....	2	5.9	82	1.19	0	3.0
Old leaf layer.....	2	5.6	80	1.58	252	20.0
Upper raw humus.....	0-4	4.3	76	1.68	388	Trace
Middle raw humus.....	4-7	3.7	59	1.23	95	Trace
Lower raw humus.....	7-9	3.6	63	1.26	32	0
Leached sand.....	15	3.8	3	0.06	0	0
Soft-pan.....	30	3.9	4	0.08	0	0
Topsoil.....	45	4.6				
Subsoil.....	90	4.8				
Subsoil.....	200	5.3				
<i>Spruce-mull:</i>						
Needle layer.....	1	4.7	61	1.15	336	75.0
Mull layer.....	0-2	4.3	37	0.82	80	26.0
Upper topsoil.....	8	4.1	6	0.15	2	5.0
Topsoil.....	22	4.2	4	0.08	0	1.0
Topsoil.....	35	4.3				
Topsoil.....	50	4.5				
Topsoil.....	65	4.8				
Subsoil.....	100	5.2				
<i>Spruce-raw humus (Moss):</i>						
Moss and needle layer.....	4	4.3	77	1.47	462	7.5
Upper raw humus.....	0-5	3.6	76	1.42	115	0.8
Middle raw humus.....	5-8	3.5	70	1.18	32	Trace
Lower raw humus.....	8-10	3.5	49	1.06	21	0
Leached sand.....	20	3.6	2	0.04	0	0
Soft pan.....	30	3.7	6	0.11	2	0
Topsoil.....	40	3.9				
Topsoil.....	60	4.7				

soil is due to the presence of toxic substances, which are different in the case of these two processes. Some of the ether-soluble extract of the humus material which was unfavorable to *Azotobacter* was favorable to nitrifying bacteria, and *vice versa*. These substances are destroyed

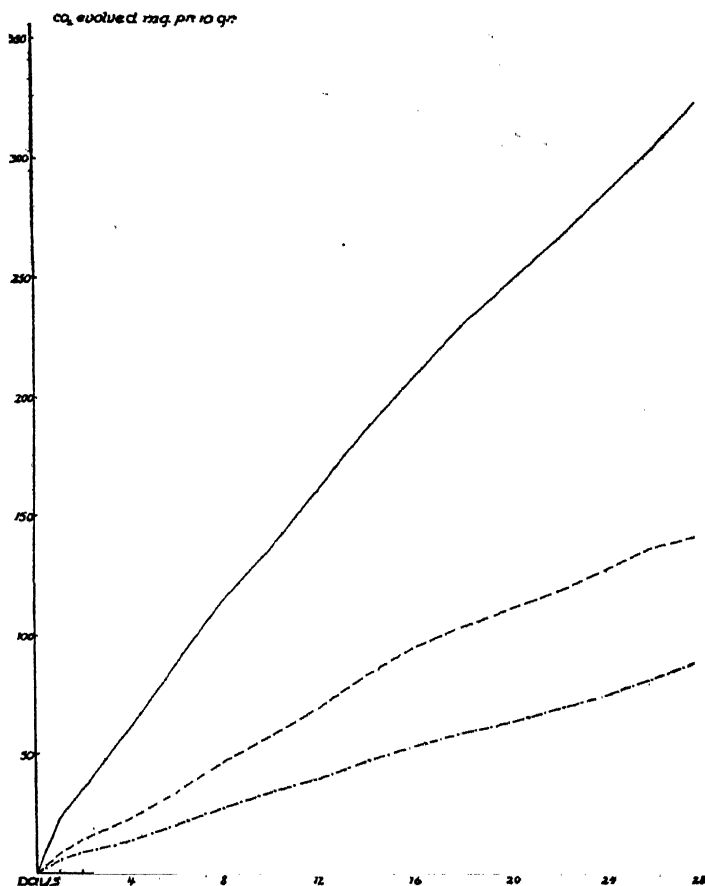


FIG. 64. Activities of microorganisms in fresh litter (—), F-horizon (-----) and H-horizon (— · — · —) from a *Pinus strobus* forest soil, as expressed by CO<sub>2</sub>-evolution (from Melin).

in garden soils by bacteria; the addition of good garden soil to forest soil removed the factor injurious to nitrification; this was believed to be due to the introduction of the bacteria capable of destroying the toxic material.

Raw-humus soils offer excellent conditions for the growth of mycotrophic plants. The host plant is enabled to take up considerably larger quantities of nitrogen and other nutrients from a limited area. Mycotrophic plants, as beech trees, have a considerable advantage over plants that do not form any mycorrhiza. The mechanism of decomposition of the organic residues in forest soils by mycorrhiza fungi and their rôle in the nutrition of the trees is still a matter of dispute (p. 269).

*Carbon dioxide evolution in forest soils.* The evolution of carbon dioxide as a result of decomposition of the organic matter in forest soils has been made the subject of a number of investigations.<sup>45</sup> In some cases, the concentration of CO<sub>2</sub> in the atmosphere has been determined, while

TABLE 95  
*Bacterial activities in different forest soils*

BACTERIAL NUMBERS OR ACTIVITIES	BEECH FOREST	ALDER FOREST	PINE FOREST
Reaction, pH.....	5.2	4.0-4.1	4.2
Moisture, per cent.....	34	56	2.1
Organic matter, per cent.....	4.2	8.6	0.5
Aerobic bacteria, agar plate.....	5,500,000	3,200,000	1,650,000
Anaerobic bacteria (shake tube)...	3,000,000	5,000,000	500,000
Aerobic nitrogen-fixing bacteria....	10		
Anaerobic nitrogen-fixing bacteria.	10,000	1,000	100
Nitrifying bacteria.....	10	10	
Aerobic cellulose-decomposing bacteria.....	1,000	100	1,000
Anaerobic cellulose-decomposing bacteria.....	10,000	1,000	1,000
Urea bacteria.....	1,000,000	100,000	10,000
CO <sub>2</sub> production.....	8.70	2.37	2.

in others the amount of CO<sub>2</sub> produced by a given quantity of soil in a definite period of time has been measured. The CO<sub>2</sub> content of forest air has been found<sup>46</sup> to depend largely upon the CO<sub>2</sub> production in the soil.

Soils of deciduous forests show a higher CO<sub>2</sub> content than soils of coniferous forests. However, when the forest soil is marshy, the development of anaerobic bacteria will result in a low liberation of CO<sub>2</sub>.

<sup>45</sup> Meinecke, Th. Die Kohlensäureernährung des Waldes. Springer. Berlin. 1927; Romell, L. G. Meddel. Statens Skogsf., 19: 125. 1922; 24: 1-56. 1923.

<sup>46</sup> Fehér, D. and Sommer, G. Erdes. Kiserl., 30: 231-266, 292-305. 1928; Biochem. Ztschr., 199: 253-271. 1928.

The temperature has a very important influence in regulating the  $\text{CO}_2$  production. The rapidity of evolution of  $\text{CO}_2$  is much greater in the decomposing horizons (F layer) of forest soils than in the humus horizons (H layer); it is much more rapid in the leaf litter than in the F layer, provided the water content and temperature are the same, as shown in fig. 64.<sup>47</sup>

Melin found that, within a given species of plants there was a distinct parallelism between the total nitrogen content in the residues and the rate of their decomposition. Some types of litter decompose more rapidly (*Fraxinus americana*, *Betula papyrifera*) and others more slowly (*Abies balsamea*). The age of the leaves and needles, mode of accumulation have also an important influence upon the rate of their decomposition.

The influence of forest type upon the abundance of bacteria in soil, as compared with the evolution of  $\text{CO}_2$ , is reported in table 95.<sup>48</sup>

<sup>47</sup> Melin, E. Särtr. Skogshögs. Festskr. 1928, 759-794; Ecology, 11: 72-101. 1930; see also Helbig, M. and Yung, C. Allg. Forst. u. Jagd. Ztg., 105: 336-344, 382-392. 1929.

<sup>48</sup> Fehér, D. Flora, 21: 316-333. 1927.

## CHAPTER XXVIII

### MICROBIOLOGICAL ANALYSIS OF SOIL, AS DETERMINED BY BIOLOGICAL, CHEMICAL, AND PHYSICAL SOIL PROPERTIES

*The soil and the activities of the microbes.* The crop-producing power of a soil is influenced by the physical and chemical conditions of the soil, such as texture, moisture content, aeration and presence of nutrient elements essential for plant growth; the availability of these nutrients is directly or indirectly affected by the activities of the soil microorganisms. By modifying the soil, as by manuring, liming and cultivation, the numbers and activities of the microorganisms are modified; this is followed by a modification of the availability of plant nutrients.

When the soil is kept well aerated and properly limed, the development of *Azotobacter*, of nitrifying bacteria, of cellulose-decomposing bacteria, and of most strains of *Bact. radicicola* is stimulated. When the soil is more acid than pH 6.0 and when it is not properly aerated, due to excess of moisture or compactness, *Cl. pastorianum* is found to predominate, as well as various nitrate-reducing bacteria, which are favored by anaerobic soil conditions. By adding an abundance of organic matter low in nitrogen to the soil, as well as by introducing fertilizers which tend to make the soil reaction acid, the development of fungi is favored, over and above that of bacteria and actinomyces. Nitrification and denitrification, decomposition of proteins with the liberation of ammonia, or utilization of the latter by microorganisms with the building up of proteins, reduction of the supply of available nitrates in the soil, sulfate reduction and oxidation of sulfur to sulfates, are among the phenomena which can thus be controlled by soil management. The development of various plant parasites is also greatly influenced by specific soil treatment.

By heating the soil or by treating it with various disinfectants, its physico-chemical condition, as well as the nature of its flora and fauna, are affected in such a manner as to bring about increased activities of certain groups of microorganisms; this is accompanied by a greater decomposition of the soil organic matter, and results in the liberation of nitrogen in an available form and in an increased crop growth. Whether insufficient growth of cultivated plants in the so-called "sick"

or "exhausted" soils is due to the development of parasitic fungi, or of organisms competing with the soil bacteria for the plant food, or of organisms actually destroying the bacteria, or whether this is due to the formation of toxic substances by microorganisms in the soil,—there is no doubt that, by modifying the microbial flora and fauna, through heat or treatment with antiseptics, more favorable conditions for plant growth will result.

In considering the soil microflora as a whole, can a group of methods be suggested, which would supply the information necessary for an understanding of the various processes carried on by microorganisms in soil, so as to obtain an insight into the actual or potential crop-producing capacity of the particular soil? The methods for measuring the biological activities of the soil should be quantitative, for the study of numbers of all or of certain specific types of microorganisms and for measuring their physiological activities, either in the untreated soil or under specifically controlled laboratory conditions. Since soil productivity is affected, aside from the biological activities, also by the physical and chemical soil conditions, nature of crop, weather, etc., the results should not be expected to represent a mathematical function of the potential crop productivity of a given soil. The results obtained from the study of one group of soils may not necessarily apply to other soils, under different climatic, topographic and other conditions. The mechanical composition, reaction, presence of free salts, as well as the physico-chemical condition of the soil, are of great importance in this connection.<sup>1</sup>

*Methods for determining the microbiological condition of the soil.* According to Niklewski,<sup>2</sup> the value of microbiological methods for soil characterization is largely due to the fact that we are able to determine the properties of a soil more exactly than by ordinary methods of chemistry and physics. Christensen<sup>3</sup> also stated that the microbiological condition of the soil, consisting of a knowledge of the qualitative and quantitative composition of its microflora and microfauna, can be considered as an expression of its complex chemical and physical nature. Other investigators<sup>4</sup> also came to the conclusion that the measurement of the activities of soil microorganisms ( $\text{CO}_2$  evolution, nitrate formation,  $\text{NH}_3$ -formation and nitrogen-fixation) offers, in the reclamation of al-

<sup>1</sup> Löhnis, F. Landw. Jahrb., **42**: 751-765. 1912.

<sup>2</sup> Niklewski, 1912 (p. 704).

<sup>3</sup> Christensen, 1915 (p. 507).

<sup>4</sup> Barnes, J. H. and Ali, B. Agr. Jour. India, **12**: 368-389. 1917.

kali soils, a useful and quick method of obtaining an equivalent valuation of the osmotic pressure of the soil solution; it eliminates the necessity of a lengthy and cumbersome analysis and the measurement by physical methods of the osmotic pressure of the salts at varying dilutions which occur under field conditions.

Chester<sup>5</sup> was the first to suggest a formula for determining the coefficient of "zymotic efficiency," which would express the results of a quantitative-qualitative bacteriological analysis of soil. "Zymotic efficiency" was looked upon as a compound involving a number of factors, capable of individual expression. Large numbers of bacteria combined with great activities would show a high efficiency, while low efficiency would mean small numbers and low activity. The lack of proper bacteriological methods, combined with an insufficient knowledge of the various groups of soil organisms, prevented Chester and those following soon after from developing this idea.

Following the work of Chester, the study of soil microbiological activities was divided along two main lines: (1) an intensive study of numbers and types of microorganisms occurring in the soil, and (2) a study of the chemical changes taking place when a small amount of soil is added to sterile or unsterile solutions of definite composition, or when a definite chemical substance is added to a definite amount of soil and the transformations taking place are determined, after a definite period of incubation.<sup>6</sup> The principle of the original solution method consisted in inoculating various solutions, the composition of which depended upon the transformation under consideration, with comparatively large amounts of soil and determining, at the end of a definite period of incubation, the chemical change that has taken place. When a 1 per cent peptone solution is inoculated with ten per cent of soil, and the ammonia formed determined after 4 to 8 days incubation at 20°C., a correlation might be obtained between the amount of ammonia thus formed and the productivity of certain soils. Löhnis laid down two considerations which could be observed in carrying on these experiments:

1. The course of various transformations should be demonstrated quantitatively.
2. The composition of the soil solutions should be of such a nature that the transformations take place within a definite length of time; these should not

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<sup>5</sup> Chester, F. D. *Del. Agr. Exp. Sta. Rpt.*, 14: 52-66. 1903; *Bul.* 65. 1904.

<sup>6</sup> Remy, Th. *Centrbl. Bakt.* II, 8: 657, 699, 728, 761. 1902; *Landw. Jahrb. Ergänzb.*, 4: 31. 1906; Ehrenberg, P. *Landw. Jahrb.*, 33: 1-139. 1904; Löhnis, F. *Mitt. landw. Inst. Leipzig, H.* 7: 1-103. 1905.



be too rapid, so that the original differences, due to bacterial activities, should not become obliterated. On the other hand, definite transformations should take place at least within one month.

The soil method was soon substituted<sup>7</sup> for the solution method, for the biochemical soil investigations. Here, the substance is added to a certain amount of soil, well mixed and kept at optimum moisture and temperature, for a certain period, at the end of which the chemical change produced is determined.

#### NUMBERS OF MICROORGANISMS IN THE SOIL

The direct microscopic method has not as yet been sufficiently developed to be readily used for determining the total numbers of microorganisms in the soil. So far we have to depend on the plate method with all its numerous limitations, for the information on the relative abundance of microorganisms. Synthetic media should be employed for the soil bacteria and actinomyces, using 8 to 10 plates for each soil sample and taking at least 4 or 5 composite samples from each field or plot. The relative abundance of fungi can be determined on the same samples of soil, using an acid medium and a lower dilution than that employed for the determination of bacteria (one-tenth to one-hundredth).

Treatment of soil which brings about differences in fertility also results in decided differences not only in the total number of microorganisms in soil, but also in the relation between the different groups of organisms. The system of cropping and the nature of the crop grown are of importance in this connection. Engberding,<sup>8</sup> however, found that whenever a difference was observed as due to cropping, it could be accounted for by a difference in the moisture content of the soil. The addition of organic matter to the soil, either in the form of stable manure, green manure and plant stubble, is known to have a decided stimulating effect upon the number of microorganisms. After most of the available energy material has been decomposed, the numbers begin to fall again, so that in a few months the level of the control may be reached. Soluble inorganic nitrogen salts and minerals also exert a stimulating effect upon the numbers of microorganisms.

<sup>7</sup> Stevens, F. L. and Withers, W. A. *Centrbl. Bakt.* II, **23**: 355-373, 776-785. 1909; **25**: 64-80. 1910; Lemmermann, O., Fischer, H., Kappen, H. and Blanck, E. *Landw. Jahrb.*, **38**: 319. 1909; Koch, A., and Pettit. *Centrbl. Bakt.* II, **26**: 335-345. 1910; Vogel. *Ibid.*, **27**: 593-605. 1910; Lipman, J. G. and Brown, P. E. *Ibid.*, **26**: 590-632. 1910.

<sup>8</sup> Engberding, 1909 (p. 14).

The correlation between the numbers of microorganisms and soil productivity has met, on the one hand, with a certain amount of criticism, but, on the other hand, has yielded some very interesting results in the hands of a number of investigators. Löhnis<sup>9</sup> did not consider the determination of numbers of microorganisms as bearing directly upon soil processes. Certain other investigators<sup>10</sup> could not find any correlation between crop productivity and results obtained by physiological methods (ammonification, nitrification).

On the other hand, considerable confidence was placed<sup>11</sup> in the determination of numbers of microorganisms in soil by the plate method. It was believed that an insight into some of the differences in the productivity of different soils could thus be obtained. A definite correlation was actually reported to exist<sup>12</sup> between crop yield, oxidizing power of soil, nitrate production and numbers, but not between crop yield and ammonia accumulation. The relative numbers of bacteria, actinomyces and fungi can further throw light upon the chemical condition of the soil, such as soil reaction, degree of decomposition of organic matter, etc. A comparison between crop yields of a series of plots, fertilized in the same manner for a number of years, in which definite differences in fertility have been established, with bacterial numbers, is given in fig. 68. The plots have been fertilized as follows:

The A's were unlimed, the B's limed, receiving two tons of ground limestone per acre every five years; plots 4 and 19 received minerals only (640 pounds acid phosphate and 320 pounds potassium chloride per acre); plot 5 received manure (32 tons per acre) and minerals; plot 7, no fertilizer; plot 9, sodium nitrate (320 pounds per acre) and minerals; plot 18, manure, minerals and sodium nitrate.

The addition of lime had a more stimulating effect upon the numbers of microorganisms than upon crop yield. It brought about, in the particular plots, a change in reaction from one below the acid limit for the development of *Azotobacter* and not very favorable for the development of actinomyces to a reaction very favorable for the growth of both groups of organisms. This, combined with the redistribution of the various groups of soil organisms, as a result of liming, may account for the lack of perfect parallelism between the unlimed and limed soils in regard to numbers and crop yields.

<sup>9</sup> Löhnis, F. Landw. Jahrb. **42**: 751-765. 1926.

<sup>10</sup> Maassen and Behn. Arb. Biol. Reichsanst. Land u. Forstw., **11**: 399-505. 1923.

<sup>11</sup> Fischer, H. Centrbl. Bakt. II, **23**: 144-159. 1909.

<sup>12</sup> Neller, J. R. Soil Sci., **10**: 29-39. 1920; Noyes, H. A. and Conner, S. D. Jour. Agr. Res., **16**: 27-42. 1919; Waksman, S. A. Soil Sci., **14**: 321-346. 1922.

Two plots, each one-twentieth of an acre in size may be compared. Plot 9A received yearly applications of 320 pounds of sodium nitrate per acre, 640 pounds of acid phosphate and 320 lbs. of potassium chloride. Plot 11B received an equivalent amount of ammonium sulfate and the same minerals; two tons of lime were also applied every five years. The results obtained justify the conclusion that crop yields and numbers of microorganisms are parallel and are equally affected by soil treatment.

	PLOT 9A	PLOT 11B
Total bacteria and actinomyces.....	10,113,000	9,500,000
Actinomyces, per cent.....	25	26.5
Fungi (on acid medium).....	46,450	39,100
Reaction of soil, pH.....	5.5	5.8
Total crop yield, pounds.....	50,488	53,826

#### NITRIFYING CAPACITY OF THE SOIL

Several methods are available for the study of nitrification:

1. Solution or sand method. A standard sterile solution containing a certain amount of ammonium sulfate and  $\text{CaCO}_3$  or  $\text{MgCO}_3$  as a base, in addition to the necessary minerals, is placed in a series of flasks and inoculated with 10 per cent of the soil to be tested. The flasks are incubated at  $28^\circ$  to  $30^\circ\text{C}$ . for thirty days and the nitrates formed are determined by the phenol-disulfonic acid method. The results obtained by this method supply information as to the presence or absence of nitrifying bacteria, influence of stimulating substances present in the soil, etc. The solution method may be replaced by the sand method. One hundred-gram portions of pure washed sand, containing 210 mgm.  $\text{CaCO}_3$  are placed in 250 cc. Erlenmeyer flasks; 15 cc. portions of a mineral solution (2 grams  $\text{K}_2\text{HPO}_4$ , 1 gram  $\text{MgSO}_4$ , 0.4 gram  $\text{FeSO}_4$  in 1000 cc. of water) are then added to each flask. The flasks are plugged and sterilized in the autoclave for 1 hour; 5 cc. portions of a sterile aqueous solution containing 30 mgm. of nitrogen, in the form of ammonium sulfate, are added after sterilization; these are inoculated with 10 grams of soil and incubated for 30 days at  $28^\circ$  to  $30^\circ\text{C}$ . The nitrates are then determined.

2. Nitrification of the soil's own nitrogen. This consists in incubating, for 30 days at  $25^\circ$  to  $28^\circ\text{C}$ ., 100-gram portions of the soil to be tested. The soil is placed in covered tumblers and contains the optimum amount of water (50 to 60 per cent of saturation). The results obtained by this method indicate the condition of the forms of nitrogen present in the soil and the rapidity with which these are transformed into nitrate nitrogen.

3. Nitrification of ammonium sulfate. Thirty milligrams of nitrogen in the form of ammonium sulfate are added to 100-gram portions of soil, which are placed in tumblers or flasks and kept at optimum moisture for 30 days at  $25^\circ$  to  $28^\circ\text{C}$ . The nitrates formed supply information on the buffer content of the soil and on the maximum nitrate accumulation when no basic substances or buffering agents are added. Initial and final hydrogen-ion concentrations of the soil should be determined.

4. Thirty milligrams of nitrogen as ammonium sulfate and 210 mgm.  $\text{CaCO}_3$  are added to the soil. The lime should be well mixed with the soil before the ammonium sulfate is added. This amount of carbonate is equivalent to an addition of the theoretical amount of base necessary for the complete neutralization of all the nitric and sulfuric acids formed from the complete oxidation of the 30 mgm. of nitrogen, in the form of ammonium sulfate. The results obtained by this method are more indicative of the nitrifying condition of the soil than by any of the other methods, since nitrification is tested here with the reaction factor eliminated. Further work may, however, lead to a modification of this method. Initial and final hydrogen-ion concentration should always be determined.

5. Nitrification of organic nitrogenous materials. One-quarter per cent of organic matter with a high nitrogen content (10 to 12 per cent), such as dried blood, or 0.5 to 1.0 per cent of organic materials of a low nitrogen content (cottonseed meal, soy bean meal, alfalfa meal) should be employed. Nitrates are determined at the end of 15 and 30 days incubation at  $25^\circ$  to  $28^\circ\text{C}$ . The nitrate content of the original soil should always be determined.<sup>13</sup> Löhnis and Green<sup>14</sup> called attention to the fact that many of the known critical factors in solution studies on nitrification were ignored by those who have criticized them severely.

A definite correlation between the nitrifying power of a soil and its crop productivity has been observed by various investigators,<sup>15</sup> as shown in table 96 and in figures 66 and 67.

Arrhenius<sup>16</sup> utilized the results obtained from the study of the nitrifying capacity for the determination of the fertilizer requirements of sugar-cane soils. A low nitrifying capacity was found to indicate a good response to the addition of inorganic nitrogen and vice versa. He recommended, therefore, that a rationally planned system of soil management on a sugar cane plantation should include the making of a map showing the distribution of the soil's nitrifying power.

Some investigators reported that the nitrifying capacity of a soil may or may not correlate with its crop producing power and that continuous cropping, especially without fertilization, reduces the nitrifying capacity

<sup>13</sup> Waksman, S. A. *Soil Sci.*, **15**: 241-260. 1923.

<sup>14</sup> Löhnis and Green, 1914 (p. 612); see also Gutzeit, E. *Centrbl. Bakt.* II, **16**: 358-381. 1906; Buhler and Fickendey. *Ibid.*, **16**: 399-405. 1906.

<sup>15</sup> Gainey, P. L. *Soil Sci.*, **3**: 399-416. 1917; Lipman, C. B. *Proc. Soc. Prom. Agr. Sci.* 35th Ann. Med. 1914, 33-39; *Cal. Agr. Exp. Sta. Bul.* 260, 107-127; Given, G. B. *Penn. Agr. Exp. Sta. Rpt.* 1912-13, 204-206; Brown, P. E. *Centrbl. Bakt.* II, **35**: 234-272. 1912; *Jour. Agr. Res.*, **5**: 855-869. 1916; Burgess, P. S. *Soil Sci.*, **6**: 449-462. 1918; Ashby, S. F. *Trans. Chem. Soc.*, **85**: 1158-1170. 1904; Kellerman, K. F. and Allen, E. R. *U. S. Dept. Agr., Bur. Pl. Industry, Bul.* 211. 1911; Waksman, S. A. *Soil Sci.*, **16**: 55-67. 1923.

<sup>16</sup> Arrhenius, O. *Arch. Suikerind. Nederl. Ind. No.* 15. 1928; see also Alicante, M. M. Phillip. *Jour. Sci.*, **32**: 1-28. 1927.

of the soil.<sup>17</sup> While nitrification is a valuable and essential asset in fertility, it probably does not, under normal conditions, become a limiting factor in productivity. This is suggested on the basis of the fact that all normal cultivated soils contain active nitrifying organisms, which transform ammonia into nitrate.

There are cases on record where the nitrate and ammonia formation in soil and bacterial numbers are not correlated. When fresh organic

TABLE 96  
*Nitrifying capacity and soil productivity*

PRODUCTIVITY OF SOIL		NITRIFYING POWER
Ashby (Rothamsted):		
Most productive.....		93
Intermediate.....		38
Poorest.....		26
Kellerman and Allen (Nevada):		
Very productive.....		54
Productive (Pl. 40).....		20
Productive (Pl. 190).....		36
Productive (Pl. 290).....		30
Poor (Pl. 10).....		4
Poor (Pl. 30).....		3
Poor (Pl. 180).....		5
Burgess (Hawaii)		
PRODUCTIVITY OF SOIL	NITRIFYING POWER	
	Dried blood	Alfalfa meal
Best.....	20.8	15.2
Very good.....	15.2-20.0	9.6-12.8
Poorer.....	4.0-13.6	7.2- 9.0
Poorest.....	4.0	4.5

matter, particularly of a non-nitrogenous nature, is added to soil, there is a rapid increase in the number of microorganisms. This is not accompanied by an immediate increase in the amount of ammonia or nitrate in soil, but rather by a decrease, due to the fact that the microorganisms use up the available nitrogen compounds in the process of growth and multiplication. Russell and Appleyard also observed that

<sup>17</sup> Allen, E. R. and Bonazzi, A. Ohio Agr. Exp. Sta. Tech. Bul. 7. 1915.

the curve for nitrate always lags behind that of bacterial numbers. As a result of partial sterilization of soil, the bacterial numbers greatly increase without any corresponding increase in nitrates; the ammonia increases but not necessarily in proportion to the numbers. The lack of correlation between certain bacterial processes, such as nitrogen changes, and soil fertility may be due to the fact that, in these cases, the latter is limited by some factor other than the nitrogen supply, such as moisture, temperature, aeration.

Whenever plant growth is limited by the supply of compounds produced by bacterial activities, the relationship between bacterial numbers and activities and plant growth is definite. Otherwise it may be accidental or it may not exist at all.

It has also been found<sup>18</sup> that nitrites may occur in large quantities in cultures, both with and without the addition of ammonium salts, used for nitrification studies. Soils which have a low capacity for forming nitrates may produce large quantities of nitrites; the latter process is favored by the addition of magnesium and calcium carbonates.

#### CARBON DIOXIDE EVOLUTION

The carbon dioxide produced by microorganisms in the decomposition of organic matter has both a chemical and physical action upon the soil. It renders certain insoluble soil minerals soluble and it imparts to the soil, after spring plowing and cultivation, a condition of ripeness ("Gare" in German). An increased carbon dioxide production also stimulates plant growth. Since all heterotrophic aerobic microbiological processes are accompanied by the production of carbon dioxide, this can be readily taken as an index of the microbiological activities in the soil. After harvesting a crop of rye, oats, clover or alfalfa, considerable amounts of organic matter are left in the soil, so that the quantities of carbon dioxide formed, as a result of the decomposition of these residues in the soil, are quite appreciable. The larger part of the organic matter is decomposed in the first few days, the rate of decomposition soon falling off.

In 1905 Russell<sup>19</sup> pointed out that soil oxidation, when measured by the amount of oxygen absorbed, varied with the fertility of the soil; he suggested using the former as a measure of the latter. Oxidation was influenced by soil temperature, moisture and content of calcium

<sup>18</sup> Fraps, G. S. and Sterges, A. J. Bull., 412. Texas Agr. Exp. Sta. 1930.

<sup>19</sup> Russell, 1905 (p. 608).

carbonate. Stoklasa<sup>20</sup> placed 1 kgm. portions of sieved soil in a glass cylinder through which a current of air was passing at the rate of 10 liters in 24 hours; for the study of anaerobic activities, an atmosphere of hydrogen was employed. The determination of carbon dioxide evolved by a soil under given conditions of moisture, temperature and time, was found to furnish a reliable and an accurate method for the determination of bacterial activities in soil. The presence of organic matter and a favorable temperature were found to be of greatest importance in the production of carbon dioxide. Evolution of carbon dioxide occurred most abundantly in neutral or slightly alkaline soil, well aerated and abundantly supplied with readily assimilable plant nutrients; it also ran parallel with nitrification. Rahn<sup>21</sup> used sugar solutions containing  $\text{CaCO}_3$ , so that he measured not only  $\text{CO}_2$  formed by bacteria but also that produced from the interaction of the organic acids formed with the  $\text{CaCO}_3$ . Drying of soil exerted a decidedly favorable influence.

Van Suchtelen<sup>22</sup> placed upon the bottom of a jar pure sand and upon it 6 kgm. of soil. Through this he passed air, usually 16 liters in 24 hours. The intensity of  $\text{CO}_2$  production was found to be much greater at the beginning of the experiment but it decreased rapidly after a short time. Carbon dioxide was measured until a definite intensity has been attained. The average production of carbon dioxide in a unit of time was used as a measure. It was concluded that the comparison of the carbon dioxide production of different soils furnishes a better means for the estimation of their relative bacterial activities than the bacterial content. Soil cultivation was found to have a favorable influence upon the carbon dioxide evolution, well sieved soils producing 177 per cent as much  $\text{CO}_2$  as unsieved soils. An increase in aeration brought about an increase in  $\text{CO}_2$  production; moisture was found to be one of the most important factors.

The curves for bacterial numbers, nitrate content and carbon dioxide in the soil air were found<sup>23</sup> to be sufficiently similar to justify the view that all these phenomena are related. A rise in bacterial numbers was accompanied by a rise in the  $\text{CO}_2$  in the soil air, and somewhat later by

<sup>20</sup> Stoklasa, J. and Ernest, A. *Centrbl. Bakt.* II, 14: 723-736; *Ztschr. Zuckerind. Böhmen.*, 31: 291-401. 1911; Stoklasa, J. *Ztschr. Landw. Versucht. Oesterreich.*, 14: 1243-1279, 1911; *Chem. Ztg.*, 46: 681-683. 1922.

<sup>21</sup> Rahn, O. *Centrbl. Bakt.* II, 20: 38-61. 1908.

<sup>22</sup> Van Suchtelen, F. H. H. *Centrbl. Bakt.* II, 28: 45-89. 1910.

<sup>23</sup> Russell, E. J. and Appleyard, A. *Jour. Agr. Sci.*, 7: 1. 1915.

a rise in the nitrate content. The rate of decomposition of organic matter in soil was, therefore, looked upon as a function of bacterial activity. It was further demonstrated<sup>24</sup> that the principal factors affecting carbon dioxide production are, in order of importance, temperature, moisture, dissolved oxygen and the growing crop. Rao. S.

In all the investigations up to 1915, the air was either drawn through the soil, thus greatly accelerating microbiological activities, or intermittently over the soil. Potter and Snyder<sup>25</sup> found that field results can be most closely duplicated in laboratory studies when the air is

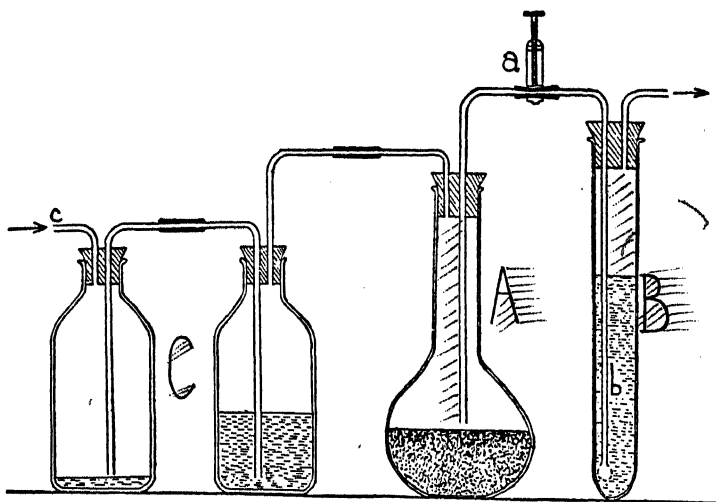


FIG. 65. Apparatus for determining the ability of soil to decompose organic matter (from Waksman and Starkey).

passed continuously over the soil. The amount of air passing over the soil does not affect materially the amount of  $\text{CO}_2$  evolved. When the soil is placed under optimum laboratory conditions, the initial rise in carbon dioxide formation is soon followed by a drop, which becomes nearly a straight line. The increase in the amount of  $\text{CO}_2$  given off by the soil, when brought into the laboratory and the moisture content adjusted, was attributed<sup>26</sup> to the previous drying of the soil, which made it a better medium, both physically and chemically, for the growth of bacteria. This is no doubt correct, since in normally moist soil

<sup>24</sup> Russell, E. J. and Appleyard, A. *Jour. Agr. Sci.*, 8: 385-417. 1917.

<sup>25</sup> Potter, R. S. and Snyder, R. S. *Iowa Agr. Exp. Sta. Res. Bul.* 39. 1916.

<sup>26</sup> Klein, M. A. *Jour. Amer. Soc. Agron.*, 7: 49. 1915.



there is no such rapid drop. Previous drying of soil alters its colloidal condition to the extent of increasing the rate of oxidation. Because of this, rainfall may increase the carbon dioxide formation in field soils, due to an increase in moisture content which is more favorable for biological activities; the importance of the oxygen brought down by the rain, as suggested by some investigators, is, in this connection, of secondary consideration.

Manure stimulates  $\text{CO}_2$  production, while sodium nitrate and ammonium sulphate do not. When  $\text{CaCO}_3$  is added to acid soil, there is at first a marked increase of  $\text{CO}_2$ , due to the chemical interaction between

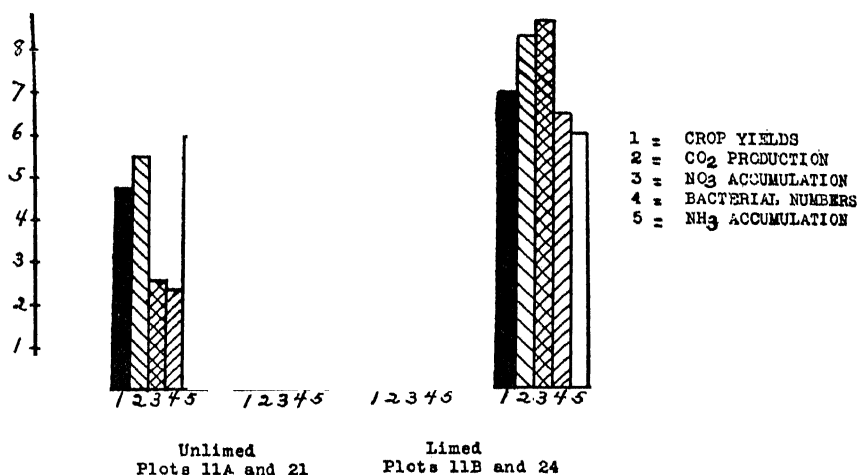


FIG. 66. The correlation between crop yields,  $\text{CO}_2$ -producing power of the soil, bacterial numbers,  $\text{NH}_3$  and  $\text{NO}_3$  accumulation in soil from limed and unlimed plots (from Neller).

the carbonate and the buffering substances of the soil. After prolonged incubation, following the addition of  $\text{CaCO}_3$ , a definite favorable effect is evidenced also upon the decomposition of soil organic matter.

A definite correlation between crop yield, nitrate accumulation and bacterial numbers, but not between crop yield and ammonia accumulation, is shown in fig. 66, where the bacterial activities of limed and unlimed soils are compared.<sup>27</sup> One can differentiate between the formation of  $\text{CO}_2$  in untreated soil and the formation of  $\text{CO}_2$  from glucose or other available organic matter added to the soil.<sup>28</sup> In the first instance,

<sup>27</sup> Neller, 1920 (p. 695).

<sup>28</sup> Waksman, S. A. and Starkey, R. L. *Soil Sci.*, 17: 141-161. 1924.

a definite amount of soil is placed under optimum conditions of moisture and temperature and the amount of  $\text{CO}_2$  formed in a definite period of time is measured. This was found by Stoklasa to depend upon (1) the number and kind of microorganisms present, (2) the amount of organic matter in the soil, (3) the composition of this organic matter and the degree of its decomposition, (4) soil aeration, (5) moisture content, (6) physical condition of the soil, (7) chemical composition (altered by fertilization), (8) soil reaction and (9) kinds of plants grown. The  $\text{CO}_2$  thus determined is a measure of the rapidity with which the soil organic matter itself decomposes under the influence of the sum total of microbiological complexes. The following procedures can, therefore, be employed:

1. One-kilogram portions of fresh soil from a composite sample taken to a depth of  $6\frac{1}{2}$  inches and put through a 3 mm. sieve are placed in pots. Enough water is then added to bring the moisture content of the soil to the optimum. The pots of soil are then placed in the respirator and the amount of  $\text{CO}_2$  evolved in fourteen days is determined at various intervals.

2. One kilogram portions of air-dried, sieved soil, taken to a definite depth, are placed in proper containers; the necessary amount of water is added and the  $\text{CO}_2$  evolved in 24 hours is determined. By this method, Stoklasa<sup>29</sup> found that an infertile soil, poor in organic matter, produced 8 to 14 mgm.  $\text{CO}_2$ , while a good beet soil produced 56 to 68 mgm.

3. One hundred-gram portions of fresh soil, prepared as in the first method, are placed in 300-cc. flasks with long necks (A in fig. 65). Cotton plugs are placed in the necks of the flasks and in the glass connections. After the proper amount of water is added (50 per cent of total moisture holding capacity), the flasks are sterilized for 1 to  $1\frac{1}{2}$  hours, on two consecutive days, at 15 pounds pressure. The soils are then inoculated with a culture of *Trichoderma* which is found to be one of the most active groups of soil fungi decomposing celluloses, proteins, pectins and other complex organic substances; a suspension of fresh cow manure may also be used for inoculation. The flasks are then connected with the  $\text{Ba}(\text{OH})_2$  tubes in the respirator and the amount of  $\text{CO}_2$  evolved is determined for 12 to 14 days. By this method, two soils, one, fertile and rich in organic matter, and another, infertile and poor in organic matter, were found to produce 124.08 and 37.40 mgm. of  $\text{CO}_2$  respectively, in eight days.

The decomposition of fresh organic matter added to the soil can be determined by a group of methods, which differ chiefly in the nature of the organic matter used. Both purified substances (glucose, cellulose) and various materials of plant and animal origin are usually employed.

A definite correlation between the numbers of bacteria and the evolution of carbon dioxide is found only in properly aerated soils. A bog

<sup>29</sup> Stoklasa, J. Compt. Rend. Agr. France, 8: 594-596. 1922.

may contain large numbers of bacteria (mostly anaerobic) but the amount of  $\text{CO}_2$  formed will be limited. On the other hand, a well aerated sandy forest soil may contain only a small number of bacteria, while the  $\text{CO}_2$ -production is very intensive. Frequently the number of certain specific soil organisms, as cellulose decomposing bacteria, is found to be proportional to the  $\text{CO}_2$ -evolution in the soil.<sup>30</sup>

#### CELLULOSE DECOMPOSING CAPACITY OF THE SOIL

Christensen<sup>31</sup> was the first to suggest that the cellulose decomposing power of a soil may serve as an index of soil fertility. A definite amount of soil was placed in Erlenmeyer flasks so as to cover four-fifths of the bottom of the flasks. Water was added from a pipette to the uncovered part of the bottom of the flask. A few strips of filter paper were then pressed upon the soil, the latter being kept moist during the period of incubation. Between 9 to 93 days were required for the complete decomposition of the paper. The physical condition of the soil and its reaction did not influence greatly its cellulose decomposing capacity. The presence of available minerals, primarily phosphates, as well as of available nitrogen were found to be of first importance; the specificity of the microbial flora must also be considered. The phenomenon of cellulose decomposition is thus influenced by the chemical and microbiological soil conditions. The amount of cellulose decomposed is governed by the available nitrogen and phosphates in the soil. Only in the case of certain peat soils, does the inoculation of the soil with cellulose decomposing bacteria have any effect.

Mütterlein<sup>32</sup> suggested placing one or two pieces of filter paper of a uniform weight (10 grams) at various depths of soil, then, after 2 to 3 weeks, removing the paper from the soil and weighing the residue; the loss in weight of the paper was taken as an index of the cellulose-decomposing capacity of the soil.

Niklewski<sup>33</sup> added cellulose to soil and measured the carbon dioxide produced. He also added to 8 kgm. of soil 1 gram  $\text{K}_2\text{HPO}_4$ , 1 gram  $\text{MgSO}_4$ , 8 grams  $\text{CaCO}_3$ , and some  $(\text{NH}_4)_2\text{SO}_4$ . The decomposition of the cellulose was chiefly controlled by the presence of available nitrogen in soil. The greater the amount of cellulose added or present in the soil, the quicker does the nitrogen need set in. Nitrogen-fixing

<sup>30</sup> Fehér, D. *Flora*, N. F., **21**: 316-333. 1927.

<sup>31</sup> Christensen, H. R. *Centrbl. Bakt.* II, **27**: 449-451. 1910; **43**: 1-166. 1915.

<sup>32</sup> Mütterlein, C. *Inaug. Diss.*, Leipzig. 1913.

<sup>33</sup> Niklewski, B. *Centrbl. Bakt.*, II, **32**: 209-217. 1912.

organisms were thought to play only a secondary rôle in normal soils, since cellulose is very slowly decomposed without the addition of available nitrogen. This would not be the case if nitrogen-fixing organisms were active, as when glucose is added. When only 0.125 per cent cellulose was added to a loess soil, containing 0.15 per cent nitrogen, a nitrogen need could be observed. The greater the amount of cellulose added, the greater was the evolution of  $\text{CO}_2$  up to a certain concentration, 1.5 per cent giving at first less  $\text{CO}_2$  than 1.0 per cent cellulose. When the available nitrogen is exhausted, the curve soon falls to a certain level depending upon the rapidity of decomposition of the nitrogenous substances in the soil and the rate with which the nitrogen become available. The addition of 0.0125 per cent ammonium sulfate greatly stimulated cellulose decomposition; this amounted, for every 10 grams of cellulose, to about 1 gram of  $(\text{NH}_4)_2\text{SO}_4$  in the case of soil and 2 grams in the case of sand. Larger concentrations of mineral nitrogen compounds acted injuriously; this injurious action may be later overcome.

On comparing the evolution of carbon dioxide, with and without the addition of a nitrogen salt, Niklewski suggested that the available nitrogen in soil can be calculated from the amount of cellulose decomposed, as indicated by the evolution of  $\text{CO}_2$ . In the case of loess soil, with a total of 0.150 per cent nitrogen, 0.040 per cent of the nitrogen was found to be active, while in the case of a sandy soil with a total of 0.015 per cent, all the nitrogen was active, or could be made readily available.

One per cent of cellulose, in the form of finely divided or ground filter paper, is added to soil sieved through a 2-mm. sieve. After carefully mixing the paper with the soil, the proper amount of moisture is added. At the end of the incubation period, the soil is air dried and the amount of cellulose left undecomposed determined. Stable manure was found to have a decided effect upon cellulose decomposition in the soil, especially when the moisture content is satisfactory, due to the nutrients present, largely the nitrogen. The greater the amount of nutrients (nitrogen and minerals) present in the manure, the greater is its favorable influence. The poorer the soil is, the greater is the influence of the manure. When ammonium sulfate and manure containing the same amount of nitrogen were added to soil, the stimulating effect upon cellulose decomposition was found to be the same.<sup>34</sup> The influence of reaction is not of great importance in cellulose decomposition.

<sup>34</sup> Charpentier, C. A. G. Inaug. Diss. Helsingfors. 1921; Barthel, Chr. and Bengtsson, N. Meddl. No. 248, Centralanst. Forsoksv. Jordbrucks. Bakt. Avdel. No. 29, 1923.

These facts, namely that the cellulose decomposing power of a soil depends more upon the physical and chemical conditions of the soil, especially the available nitrogen, rather than upon a specific microbial flora, can be readily explained when the activities of microorganisms concerned in cellulose decomposition in the soil are considered.

As pointed out above, cellulose is decomposed in normal soils largely by various fungi and aerobic bacteria. Only in soils saturated with water do the anaerobic bacteria become active in cellulose decomposition. The ratio between the amount of cellulose decomposed and the amount of nitrogen assimilated is about 30:1 in the case of pure cultures of fungi and aerobic bacteria; however, in the soil itself, when the cells of the microorganisms freshly synthesized are constantly decomposed

TABLE 97

*Influence of different forms of nitrogen upon the decomposition of cellulose in soil*<sup>34</sup>  
(1 per cent filter paper added to 1 kilogram of soil)

NITROGEN SOURCE	CELLULOSE LEFT, PER CENT		
	At start	After 2 months	After 4 months
Control.....	0.87	0.53	0.48
16 mgm. nitrogen, as manure (10 grams).....	0.80	0.51	0.31
32 mgm. nitrogen, as manure (20 grams).....	0.85	0.38	0.24
64 mgm. nitrogen, as manure (40 grams).....	0.80	0.27	0.18
32 mgm. nitrogen, as $(\text{NH}_4)_2\text{SO}_4$ .....	0.85	0.35	0.26
16 mgm. nitrogen, as $\text{NH}_4\text{NO}_3$ .....	0.83	0.34	0.27
32 mgm. nitrogen, as $\text{NH}_4\text{NO}_3$ .....	0.81	0.22	0.13

by other organisms, the ratio is 50-60:1. In other words, for every unit of nitrogen that can become available in soil in a definite period of time, about 50 to 60 units of cellulose will be decomposed. If one gram of cellulose in the form of ground filter paper is added to 100 grams of soil, incubated at optimum temperature and moisture for 30 or 60 days, and the amount of cellulose decomposed in that period of time found to be 400 mgm., it indicates that about 7 to 8 mgm. of nitrogen can become available in the given soil in the particular period of time. Table 97 brings out the influence of nitrogen in different forms upon the decomposition of cellulose in soil.

To measure the cellulose-decomposing power of soil, three methods are recommended:<sup>35</sup>

<sup>35</sup> Waksman, S. A. and Heukelekian, O. *Soil Sci.*, 17: 275-291. 1924.

1. One gram of finely cut or well ground filter paper is well mixed with 100 grams of fresh sieved soil. This is placed in a tumbler, brought to optimum moisture, covered, and incubated for 42 days, at 25° to 28°C. with frequent additions of water to keep at optimum moisture. The amount of residual cellulose is determined by the method of Charpentier in the soil which is first air dried. The residual cellulose is then subtracted from the amount of cellulose originally present in soil; this can be determined by extracting 20 grams of the original soil to which 200 mgm. of the paper has been added. The amount of cellulose actually decomposed in the soil is thus obtained.

2. One gram of well ground filter paper and 100 mgm. of sodium nitrate are added to 100 grams of soil. The mass is well mixed and placed in a tumbler, brought to optimum moisture, covered, and incubated for 15 days. The amount of cellulose decomposed is determined as in the case of the first method.

3. One hundred grams of soil, 200 mgm. of  $\text{CaCO}_3$ , 50 mgm.  $\text{K}_2\text{HPO}_4$ , 25 mgm.  $\text{MgSO}_4$ , with and without one gram of ground dry filter paper are mixed in tumblers. These are placed in a respiratory apparatus and the amount of  $\text{CO}_2$  given off in fourteen days is determined. The excess of  $\text{CO}_2$  produced in the soil containing the cellulose over that produced in the soil containing the minerals only, and the amount of cellulose decomposed will serve as an index of the cellulose decomposing power and, *ipse facto*, of the available nitrogen in the soil.

#### NITROGEN-FIXING AND MANNITOL DECOMPOSING CAPACITY OF THE SOIL

The principle of the various methods used for the study of the nitrogen-fixing capacity of soil can be summarized as follows. A readily available source of energy, chiefly mannitol or glucose, is added to soil or to a solution inoculated with soil; the amount of available nitrogen in soil is very limited, so that the fungi and heterotrophic non-nitrogen-fixing bacteria, which would otherwise be capable of consuming the mannitol or glucose, cannot do that extensively. The amount of glucose or mannitol commonly used in laboratory studies (1 to 2 per cent) is in considerable excess, so that the amount of available nitrogen is far from sufficient for supplying the requirements of the non-nitrogen-fixing organisms. The bacteria, which are capable of utilizing the gaseous atmospheric nitrogen, can readily use mannitol or glucose as sources of energy. The addition of glucose to soil brings about an abundant multiplication of bacteria, especially the nitrogen-fixing forms.

In the presence of an available source of energy, the nitrogen-fixing bacteria may become limited in their development by the lack of sufficient available phosphorus in the soil or in the medium. Since *Azotobacter* cells contain as much as 2 to 5 per cent  $\text{P}_2\text{O}_5$ , the rapid development of this and other nitrogen-fixing bacteria, which produce an extensive growth in the presence of an excess of available energy, may be limited by the presence of this mineral. For every unit of

nitrogen fixed or assimilated by *Azotobacter* and synthesized into microbial protein about half a unit of available phosphorus ( $P_2O_5$ ) is required. The amount of phosphorus present in an available form can be calculated from the amount of nitrogen fixed, provided there is sufficient energy material. The latter may then become merely an index of the available phosphorus in the soil.

Four methods may be suggested<sup>36</sup> for measuring the nitrogen-fixing and mannitol decomposing power of a soil:

1. *The solution method.* One or five grams of soil are added to 50 or 100 cc. of a standard mannitol solution (20 grams mannitol, 0.2 gram  $MgSO_4 \cdot 7H_2O$ , 0.2 gram  $K_2HPO_4$ , 0.2 gram  $NaCl$ , 5.0 grams  $CaCO_3$ , in 1000 cc. distilled water and made neutral to phenolphthalein). After 7 to 28 days incubation, the increase in total nitrogen above the control is determined (original solution + original soil is analyzed immediately for total nitrogen). This serves as an index of the activities of the nitrogen-fixing flora of the soil and thus also, to some extent, of the microbiological condition of the soil.

2. *The soil method.* One or two grams of mannitol are added to 100 grams of fresh sieved soil; the latter is brought to optimum moisture, incubated for 28 days, and the increase of nitrogen in the treated soil over the untreated soil, incubated under similar conditions, determined.

3. *The pure culture method.*<sup>37</sup> Ten grams of the particular soil are added to 100 cc. of a 2 per cent mannitol solution, free from available phosphates, sterilized and inoculated with a vigorous culture of *Azotobacter*. After incubating for 20 to 30 days, the increase in total nitrogen is determined. This can serve as an index of the available phosphate in the soil.

4. *The determination of residual mannitol* (or rather soluble organic matter in the soil).<sup>38</sup> This consists in adding 2 per cent of mannitol to air dry soil, bringing to optimum moisture, incubating, and determining the residual mannitol every five days by oxidation with  $KMnO_4$ . This method can serve as an index of the activities of the nitrogen fixing flora of the soil, as well as of the amount of phosphorus available.

The method of determination of soluble organic matter in the soil is carried out as follows. Five grams of soil are withdrawn and allowed to air-dry; the air-dry soil is then weighed again and extracted for two hours, with occasional shaking, with 200 cc. of water. The extract is filtered through paper and 10 cc., or an amount equivalent to 0.25 gram of soil, is placed in a 400-cc. beaker with 50 cc. of 0.05 *N* potassium permanganate solution and 3 cc. of dilute (6:100) sulfuric acid. The beaker is placed in boiling water for twenty minutes, 50 cc. of 0.05 *N* oxalic acid is then added and the solution is titrated with 0.02 *N* potassium permanganate solution. The number of cubic centimeters of the latter expresses the amount of organic matter (residual mannitol + soluble soil organic matter).

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<sup>36</sup> Waksman, S. A. and Karunaker, N. *Soil Sci.*, 17: 379-393. 1924.

<sup>37</sup> Niklewski, 1912 (p. 704); Stoklasa, 1925 (p. 545); Christensen, 1915 (p. 507).

<sup>38</sup> Christensen, H. R. *Soil Sci.*, 15: 329-380, 361-366. 1923.

A definite correlation was found to exist between nitrogen fixation in mannitol solution or in soil, to which an energy source has been added, and the crop-producing capacity of the soil.<sup>39,40</sup> By adding a definite amount of soil (10 grams) to a mannitol solution, free from phosphates, then inoculating with a culture of *Azotobacter* and determining the amount of nitrogen fixed, after a definite period of incubation, an approximate index of the presence or absence of available phosphorus in the soil can be obtained,<sup>40,41</sup> as shown in table 98.

TABLE 98  
*Nitrogen fixed in 100 cc. of mannitol solution + 10 grams of soil*

SOIL NEED OF PHOSPHORUS	SOLUTION STERILIZED BEFORE SOIL WAS ADDED		SOLUTION STERILIZED AFTER SOIL WAS ADDED	
	P <sub>2</sub> O <sub>5</sub> in medium	No P <sub>2</sub> O <sub>5</sub> in medium	P <sub>2</sub> O <sub>5</sub> in medium	No P <sub>2</sub> O <sub>5</sub> in medium
	mgm.	mgm.	mgm.	mgm.
None.....	8.22	5.35		2.85
Medium.....	5.48	4.08	14.51	1.95
Great.....	3.78	1.67	15.05	0.35

The available phosphorus in soil may be calculated from the amount of nitrogen fixed. To 100 grams of soil, 30 cc. of water containing 2.5 grams of glucose, 0.2 gram K<sub>2</sub>SO<sub>4</sub> and 0.05 gram of MgCl<sub>2</sub> were added. The soil was then sterilized and inoculated with *Azotobacter*. After 21 days incubation, the total nitrogen and phosphoric acid were determined in the soil. The following process was used for calculating the amount of available phosphorus. One hundred grams of soil contained 0.164 gram nitrogen in the inoculated and 0.110 gram in the uninoculated soil. The amount of nitrogen fixed was, therefore, 0.054 gram. Since *Azotobacter* cells contain 10 per cent nitrogen and 5 per cent P<sub>2</sub>O<sub>5</sub>, 0.027 gram of the latter was made available in the given quantity of soil. The total P<sub>2</sub>O<sub>5</sub> in 100 grams of soil was 0.103 gram, hence about 26 per cent of this phosphorus is readily available.<sup>42</sup> A very fertile soil containing 0.084 per cent P<sub>2</sub>O<sub>5</sub> has shown 48.8 per cent of it utilizable; a soil of medium fertility contained 26.21 per cent utilizable P<sub>2</sub>O<sub>5</sub>, and a poor forest soil only 11.66 per cent of the P<sub>2</sub>O<sub>5</sub> utilizable.<sup>42</sup>

There is no doubt that all agricultural soils can be made to fix nitrogen when an excess of an available source of energy is added. However,

<sup>39</sup> Löhnis, F. and Pillai, N. K. *Centrbl. Bakt.* II, 20: 781-795; Green, H. *Ibid.*, 41: 577-608. 1914; Burgess, 1918 (p. 697).

<sup>40</sup> Brown, P. E. *Iowa Agr. Exp. Sta. Res. Bul.* 25. 1915.

<sup>41</sup> Niklewski, 1912 (p. 704).

<sup>42</sup> Butkewitsch was the first to suggest the use of *Asp. niger* for the purpose of determining the abundance of certain nutrients in the soil. A certain quantity of soil was added to a medium lacking a definite nutrient; from the amount of



the reaction of the soil, which favors the development of specific nitrogen fixing organisms, is of great importance in this respect, as pointed out above. The presence of available phosphorus and the soil reaction influencing the development of specific nitrogen-fixing bacteria, are the two factors controlling the amounts of nitrogen fixed and mannitol decomposed.

Winogradsky<sup>43</sup> suggested several new methods for determining the nitrogen-fixing capacity of the soil, the results serving in a way as a measure of soil fertility.

1. A silica gel plate, 9 cm. in diameter, is inoculated with a few grains of soil. In the presence of *Azotobacter*, the soil will be surrounded, after 48 hours, with the colonies of the organism. The relative abundance of the colonies will indicate the biological activities of the soil.

2. One-half gram of mannitol is added to 50 grams of soil, which is incubated for 48 hours. The soil is then examined microscopically and the abundance of nitrogen-fixing bacteria serves as an index of the activity of the soil.

3. Five parts of starch are added to 100 parts of fresh soil and the mixture kneaded with sufficient water and placed in a Petri dish; an excess of water is avoided. After 48 hours incubation, minute colonies will be formed on the surface of the mixture; the abundance of the colonies serves as an index of the activity of the soil.

4. A large silica-gel plate, containing 2 grams of mannitol is inoculated with 1 gram of soil. After 48 hours incubation, the number of colonies on the plate is determined; after 5 days incubation, the contents of the plate are analyzed for total nitrogen. An active soil will show 2,000 to 3,000 colonies per 1 gram of soil and will fix 20 mgm. of nitrogen for the 2 grams of mannitol.

By the use of these methods, Winogradsky distinguished three types of soil: 1. Very active soils, characterized by an abundance of aerobic

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growth produced by *Asp. niger*, the amount of the particular nutrient present in the given quantity of soil in an available form was calculated. This method was modified by Christensen, whereby *Azotobacter* was used for determining the available phosphorus, potassium and calcium in soil. It was further extended by Stoklasa and Niklas. A comparison of the results obtained by the microbial method and the vegetation methods of Mitscherlich gave (Benecke) very encouraging results for the value of the microbiological method. Butkewitsch, W. Zhur. opit. Agron., 10: 136-141. 1909; Koszelezki, A. Ibid., 321-354; Christensen, H. Ztschr. Pflanzen. Düng. A1: 265. 1922; Niklas, H. and Hirschberger, W. Ill. landw. Ztg., 49: 379. 1924; Stoklasa and Doerell, 1926 (p. XIV). Truffaut, G. and Bezsonoff, N. La Sci. du Sol., 7. 1928; for limitations of method, see Stöckli, A. Landw. Jahrb. Schweiz., 1929: 811-840; Benecke, W. and Söding, H. Ztschr. Pflanzenern. Düng. A 10: 129-158. 1927.

<sup>43</sup> Winogradsky, S. Compt. Rend. Acad. Sci., 182: 907, 999, 1061. 1926; Ann. Inst. Past., 40: 455-520. 1926; 42: 36-62. 1928.

nitrogen-fixing bacteria and by the ability to readily form spontaneous cultures of these bacteria when enriched with an assimilable carbon source. 2. Soils only moderately active, with retarded or no development of spontaneous cultures and less abundant in nitrogen-fixing bacteria. 3. Inactive soils, free from aerobic nitrogen-fixing bacteria. The spontaneous culture of *Azotobacter* was found<sup>44</sup> to be the most convenient method for determining the available phosphorus in different soils.

#### THE CATALYTIC ACTION OF SOIL

The catalytic action, or the catalytic power, of a soil is its ability to produce oxygen from hydrogen peroxide; this has often been found to be an index of the fertility of the soil. It can be determined by adding 5 grams of soil to 20 or 40 cc. of a 1.5 or 3 per cent solution of  $H_2O_2$  and collecting the oxygen liberated; a 100 cc. gas-measuring tube filled with a dilute solution of NaOH or KOH and inverted into a bath of the same solution is used for collecting the gas. The soil is usually placed in a large test tube or in a 300-cc. Erlenmeyer flask and may be suspended in a little water, before adding the peroxide. One portion of soil is used untreated and another portion is previously sterilized in the autoclave so as to determine the rôle of the living organisms in the process; a third portion of soil may be ignited and then used in the test, so as to determine the rôle of the organic matter in the process of  $H_2O_2$  decomposition. The period of incubation is usually 5 to 60 minutes and the temperature 17° to 37°C. An increase in concentration of substrate, temperature and period of incubation all lead to an increase in the amount of oxygen liberated.

König<sup>45</sup> found that the decomposition of  $H_2O_2$  by soil was due chiefly to the enzyme catalase produced by the soil microorganisms and plant materials, and to some extent to the inorganic part of the soil and to organic colloids. On sterilizing the soil by heat or treating with chloroform, iodine, mercury bichloride and especially hydrocyanic acid, the liberation of oxygen is greatly diminished. The catalytic action was found to be further increased by a similar action of manganic oxide, iron and aluminum oxides. The formation of oxygen by heated soils was ascribed to the increase in alkalinity due to the formation of CaO

<sup>44</sup> Guittonneau, G., Keilling, J. and Béjambes, M. *Ann. Sci. Agron.*, **46**: 133-165, 255-291. 1929.

<sup>45</sup> König, J., Coppenrath, E. and Hasenbäumer, J. *Landw. Vers. Sta.*, **66**: 401-461. 1907; **53**: 472-476. 1906.

from  $\text{CaCO}_3$ . When heated soil is moistened and allowed to remain one day in a desiccator filled with  $\text{CO}_2$ , its catalytic power is greatly diminished. A direct correlation was demonstrated between the humus content of the soil and its catalytic power. According to May and Gile,<sup>46</sup> the catalytic action of a soil is a rough measure of the combined

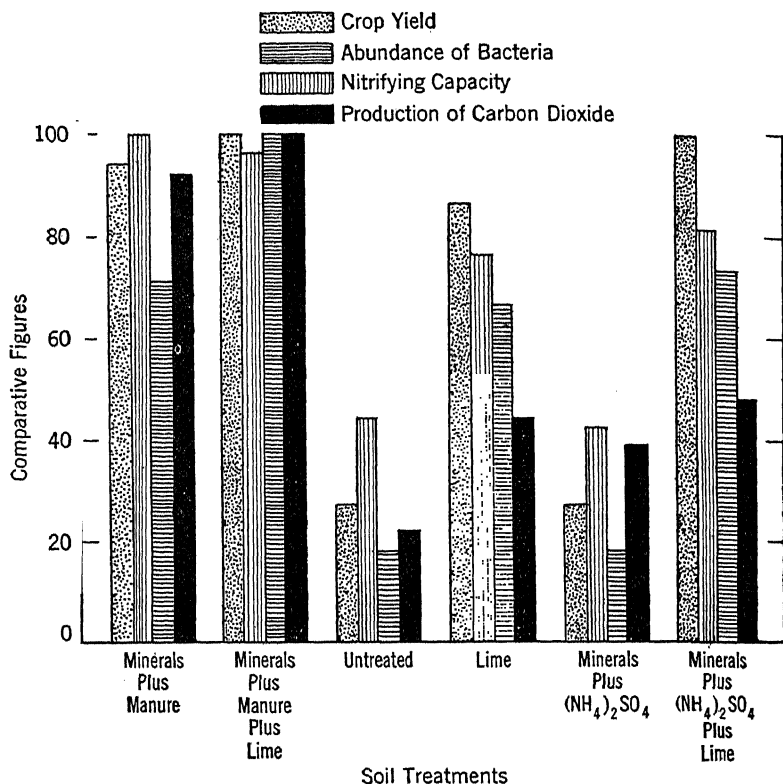


FIG. 67. Correlation between microbiological activities and crop yields of soil, originally the same, but variously treated for 15 years (after Waksman and Starkey).

quantity of bacteria and organic matter present. Surface soils were found to be more active than subsoils, more fertile soils are more active than infertile ones.

Since inorganic soil constituents are also capable of liberating oxygen

<sup>46</sup> May, D. W. and Gile, P. L. Porto Rico Agr. Exp. Sta. Circ. 9. 1909; Sullivan, M. X. and Reid, F. R. Bur. of Soils. U. S. Dept. Agr. Bul. 86. 1912.

from  $H_2O_2$ , Chouchack<sup>47</sup> used the difference between the oxygen formed by normal soil and that formed by the same amount of sterilized soil as an index of the biological activities. By treating the soil with phosphates, potassium and nitrogen salts, then determining the increase in catalytic action, reliable information can be obtained on the practical value of such treatments. Osuga,<sup>48</sup> however, confirmed the previous observations of König that ferric oxide, manganese oxide and humus show marked catalytic action. He suggested that these substances may be the main constituents which react with the  $H_2O_2$ . Bacterial effect in soil catalysis was believed to be small. In this respect, he confirmed the earlier observations of Kappen<sup>49</sup> that the catalytic action of the soil is due largely to its colloidal complexes.

The catalytic action of the soil is thus found<sup>50</sup> to be due, to (1) the inorganic constituents of the soil; (2) to certain organic soil compounds, such as benzol derivatives; (3) to the action of catalase formed by microorganisms in the soil. Although a correlation exists between the catalytic action of soil, the numbers of microorganisms, as well as soil productivity, the phenomenon is very complex and cannot be used as a simple method for measuring the fertility of the soil, unless the various factors are determined individually. According to Scharrer,<sup>51</sup> the greater the Mn, Fe and total Ca content of the soil the greater is its catalytic activity. Neutral peroxide must be used, as the catalytic power of the soil, even if not a direct function of the pH, is greater in neutral or alkaline than in acid soils. Sandy soils were found to have the least, and loam and clay soils the greatest catalytic activity. Humus soils usually give higher values than mineral soils, acid peats lower than neutral peats. Soils containing the lowest number of bacteria also possess the lowest catalytic activity, although there is no direct relation between bacterial count and catalytic activity.

#### OXIDIZING AND REDUCING POWER OF THE SOIL

Oxidation consists in the addition of oxygen or subtraction of hydrogen; the oxygen can be obtained either directly from the atmosphere

<sup>47</sup> Chouchack, D. *Compt. Rend. Acad. Sci.*, **178**: 1842-4, 2001-2. 1924.

<sup>48</sup> Osuga, C. *Ber. Ohara. Inst. Agr. Invest. Kuraschiki.*, **2**: 197-218. 1922.

<sup>49</sup> Kappen, H. *Fühling's landw. Ztg.*, **62**: 377-392. 1913; Smolik, L. *Proc. Intern. Soc. Soil Sci.*, **1**: 6-21. 1925; A detailed review of the occurrence and action of catalase in general is given by Morgulis, S. *Ergebn. Physiol.*, **23**: 308-367. 1924.

<sup>50</sup> Waksman, S. A. and Dubos, R. *Soil Sci.*, **22**: 407-422. 1926.

<sup>51</sup> Scharrer, K. *Biochem. Z.*, **189**: 125-149. 1927; *Ztschr. Pflanz. Düng. Bodenkn.*, **12B**: 323-329. 1928.

or from a peroxide. Soil fertility and the rate of oxidation were found<sup>52</sup> to be influenced by the same factors and to the same extent, so that it was suggested that the latter could be used as a measure of the former. Oxidation is greater in fertile than in infertile soils, in surface soil than in subsoil. The oxidizing power of soils can be determined<sup>53</sup> by shaking 5 grams of soil with 10 cc. of an alcoholic solution of gum guaiac and then allowing the soil to settle. The formation of a blue color indicates the degree of oxidation. When the blue color fades, it can be brought back by the addition of 0.5 cc. of a 2 per cent  $H_2O_2$  solution. Another method of testing oxidation in soils consists in shaking 20 grams of soil

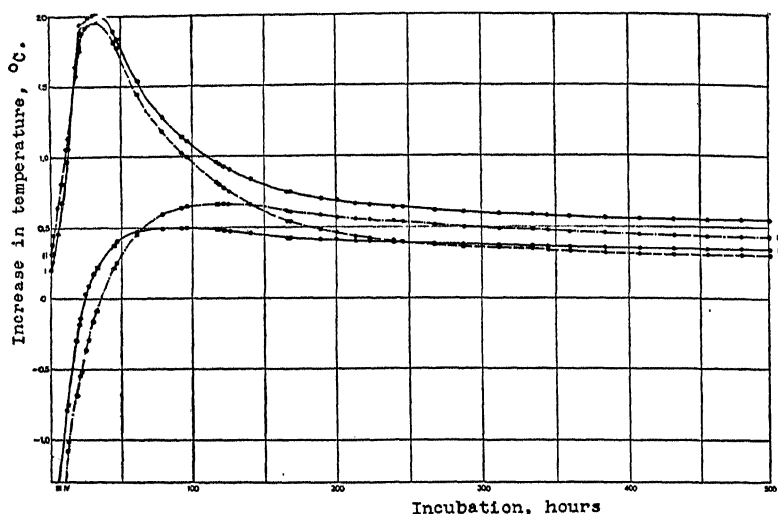


FIG. 68. Heat evolution from different soils (from van Suchtelen).

with 50 cc. of 0.125 per cent aqueous solution of aloin for one hour; the soil is allowed to settle or the solution is centrifuged (if turbid, 50 cc. of 95 per cent alcohol is added to flocculate the soil and extract the oxidized aloin); the clear solution is poured off and the depth of color is determined by a colorimeter. Soils known to be productive have a strong oxidizing power, and the poorer soils have little or no oxidizing power; factors favoring oxidation also favor soil productivity. However, agreement has not been obtained in all cases.

<sup>52</sup> Russell, 1905 (p. 608).

<sup>53</sup> Schreiner, O. and Sullivan, M. X. Bur. of Soils, U. S. Dept. Agr. Bul. 73. 1910.

Gerretsen<sup>54</sup> used, as an index of the oxidizing power of soils, the amount of iodine liberated when 100 grams of soil are treated with a dilute solution of potassium iodide acidified with sulfuric acid. He found that rich soils had a high iodine (or HI) value; poor soils had a low value. These results were not confirmed, however, by Honing<sup>55</sup> for soils of Deli, due to large quantities of organic matter present and to the irregular distribution of the ferric iron. The oxidation of sulfur in soil, the reduction of nitrates (denitrification), and the formation of ammonia from proteins are often used for comparing the microbiological condition of different soils, but the value of these methods in throwing light upon the soil microbiological processes is often questioned.

*The heat evolution from soil.* As seen from the above discussion, the whole field of soil microbiology has been regarded largely from a chemical point of view; the methods employed for the study of the microbiological status and changes in soil were accordingly mostly chemical. During recent years, however, a physical indicator has been successfully employed in this field of study by which the activity of soil microorganisms, or the intensity of their transformations, has been measured by their heat production.

The heat production measured in gram-calories, per hour per liter, may be regarded as a valuable biophysical measure in this respect. In this method, the minute quantities of heat produced were accumulated in thermos bottles, and the recorded temperature represents that heat in excess of a sterile check. The heat production was found to be stimulated during the first days of incubation, because of the increased supply of oxygen; the heat evolution becomes constant after some time, and the latter constant temperature enables one to come to a conclusion concerning the heat production of the soil (fig. 68).<sup>56</sup>

<sup>54</sup> Gerretsen, F. C. Meddl. Proefsta. Java. Suikerind., 5: 317-331. 1915; Ibid. No. 3. 1921.

<sup>55</sup> Honing, J. A. Bul. Deli Proefsta, No. 8. 1917; Bul. Agr. Intel., 8: 838. 1917.

<sup>56</sup> van Suchtelen, F. H. H. Centrbl. Bakt. II, 58: 413-430. 1923; 71: 53-72. 1927; 79: 108-123. 1929.

## CHAPTER XXIX

### SOIL MICROBIOLOGICAL EQUILIBRIUM

#### INFLUENCE OF AIR DRYING AND PARTIAL STERILIZATION OF SOIL UPON THE ACTIVITIES OF MICROORGANISMS

*Microbiological equilibrium in the soil.* The numerous types of microorganisms harbored in the soil vary greatly morphologically and physiologically. Conditions favoring the activities of one group of organisms may be distinctly injurious to others. An acid reaction, for example, may be favorable to the development of certain fungi but inhibitory to a large number of bacteria and actinomycetes. The presence of an excess of lime may favor bacteria, including nitrogen-fixing, nitrifying and other groups, but may depress the development of many fungi. Aeration has a favorable influence upon some organisms but not upon others. The soil flora is so complex and the resulting activities are so various that one group of processes can hardly be separated from another and studied by itself. The addition to the soil of nitrogen compounds, carbon compounds, or minerals does not only stimulate the activities of one or more groups of organisms, but may bring about a series of changes in the soil, the resultant or the end of which is hard to foresee. Not only are the various organisms affected in different ways by different soil treatments, but they themselves exert stimulating or injurious influences upon the activities of one another.

When a soil is left undisturbed for a long period of time the numbers and activities of the various groups of organisms come to a condition which may be termed unstable equilibrium. This equilibrium is not static but dynamic, in a chemical sense, especially under field conditions. Sunshine and rain, freezing and thawing, plowing and cultivating, fertilizing and manuring, and a host of other factors which affect the soil will bring about a change in this equilibrium. If the numbers of bacteria and protozoa are determined daily for a period of time, constant fluctuations are found.<sup>1</sup> The same is true of the numbers of fungi, nitrate and carbon dioxide content of the soil.

<sup>1</sup> Cutler, et al., 1922 (p. 29).

When a soil is kept under constant optimum conditions and undisturbed, the daily variability is very small and there is a constant gradual diminution in the numbers and activities of the microorganisms, as shown<sup>2</sup> in fig. 69. The curves indicate that the rapid rise in the numbers of bacteria and evolution of carbon dioxide, as a result of moistening of an air-dry soil, was followed by a gradual drop for about 200 days; the drop then became hardly perceptible but was still present. The soil was kept in pots and the soluble products, resulting from the decomposition of the organic matter in the soil, were not removed by drainage, nor by growing plants, nor by any microorganisms, since no

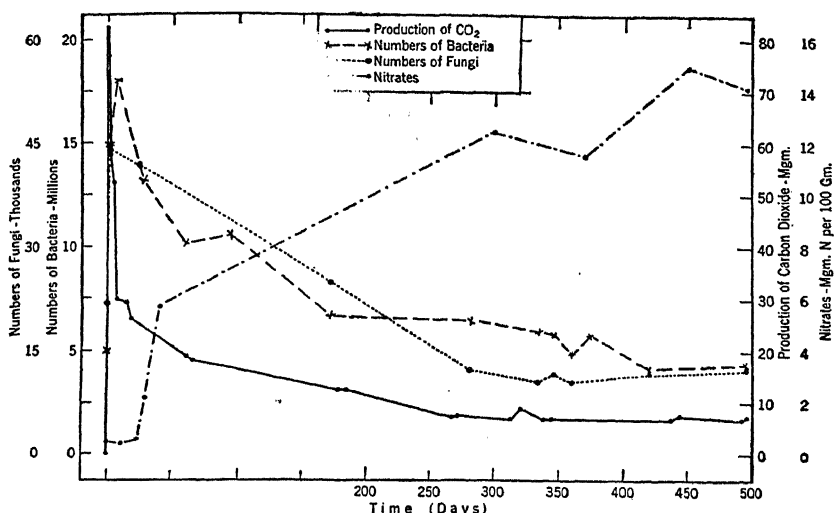


FIG. 69. Course of microbiological activities following the remoistening of a dry soil (after Waksman and Starkey).

fresh sources of energy were added. For this reason nitrates continued to accumulate. This equilibrium in microbiological activities was not due to a lack of nitrogen, but to a lack of available energy. Rahn<sup>3</sup> found that the addition of straw to such a soil will result in a rapid increase in the growth and development of microorganisms, lasting as long as the available energy does and followed again by a decline. The same increase in the activities of microorganisms can also be obtained by

<sup>2</sup> Waksman, S. A. and Starkey, R. L. *Soil Sci.*, **16**: 137-156, 247-268, 343-357. 1923.

<sup>3</sup> Rahn, O. *Ztschr. techn. Biol.*, **7**: 172-186. 1919.



treatment of soil with various volatile antiseptics, by heating or even by mere drying. The resulting activities are similar to those following the addition of a fresh supply of energy.

*Influence of air-drying of soil upon the microbiological equilibrium.* The favorable effect of drying of soil upon the growth of higher plants has been reported from various sources.<sup>4</sup> This effect was first attributed

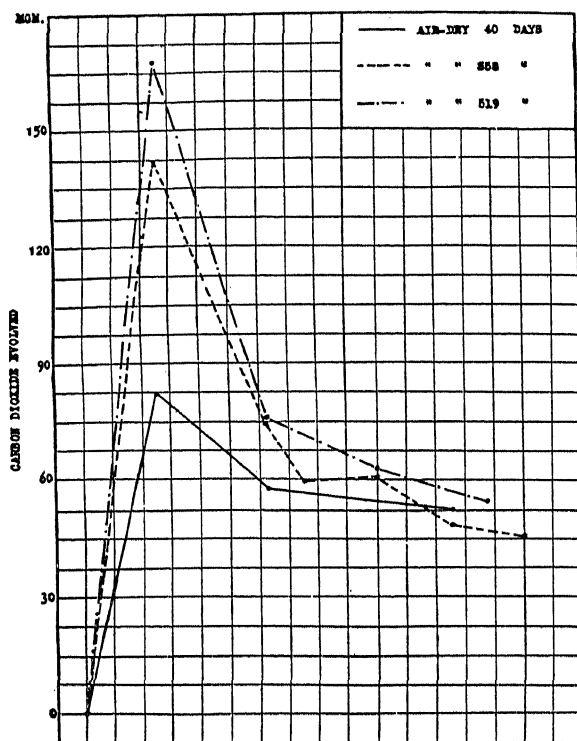


FIG. 70. Influence of length of air-drying of soil upon its biological activities, as indicated by the evolution of  $\text{CO}_2$  (from Waksman and Starkey).

to the improvement in the physical condition of the soil, especially in case of fine grained soils; it was later ascribed to some chemical changes produced in soil by drying. The numbers and activities of microorganisms are found to be also markedly influenced. When one part of soil is allowed to remain moist and the other part is air-dried for some

<sup>4</sup> Lebedjantzev, A. N. Soil Sci., 18: 419-447. 1924.

time and then moistened, a rise in the numbers of bacteria, an increase in the evolution of carbon dioxide and an accumulation of nitrates will take place in the soil that has been air-dried. The rise in the numbers and activities of microorganisms is soon followed by a rapid fall until they approach those of the control soil. When the formation of acid in glucose solution and ammonia in urea and peptone solutions were used as indices, an air-dried soil was about 20 per cent more active than the corresponding moist soil.<sup>5</sup> Different soils behave differently in this respect, heavy soils, especially garden soils, showing greater differences than light soils. The bacteriological methods of analysis were found very unsatisfactory and Rahn, therefore, ascribed the beneficial effect of drying to the degree of solubility of minerals. Soils dried gradually become more active than soils dried rapidly;<sup>6</sup> complete drying results also in a greater stimulus than moderate drying. Ritter suggested that this favorable influence of drying might be due to a selective action upon the species of soil microorganisms. The greater the period of time during which the soil is air dried, the more are the activities of microorganisms stimulated, as indicated by numbers of bacteria, evolution of carbon dioxide and increase in available nitrogen (fig. 70).

Drying of soil results in a decrease in bacterial numbers of 40 to 70 per cent, followed by a rapid increase, when the soil is moistened.<sup>7</sup> When a soil kept in pots becomes unfavorable for the growth of leguminous plants, it may be restored to normal condition by drying. The protozoa (cysts) are not destroyed as a result of drying,<sup>8</sup> neither are the fungi and actinomyces affected to any large extent.

König and associates<sup>9</sup> found that all soils except clays show a small but definite increase in dialyzable materials, when heated under diminished pressure. This is due to a change in the colloidal condition of the soil, which causes the adsorbed materials to become soluble. Air-drying of soil, under natural conditions, also causes a partial destruction of the colloidal state, and, therefore, an increase in the solubility of the nutrients held by the colloids. The influence of repeated wetting and drying of soil in increasing plant growth is believed to be due to this phenomenon.

<sup>5</sup> Rahn, O. *Centrbl. Bakt.* II, 20: 38-61. 1908.

<sup>6</sup> Ritter, G. *Centrbl. Bakt.* II, 33: 116-143. 1912.

<sup>7</sup> Heinze, B. *Landw. Jahrb.*, 55: 139-184. 1920.

<sup>8</sup> Goodey, T. *Proc. Roy. Soc. B.*, 88: 437-456. 1915; Greig-Smith, R. *Proc. Linn. Soc. N. S. Wales*, 39: 839-850. 1914.

<sup>9</sup> König, J., Hasenbäumer, J. and Glenk, K. *Landw. Vers. Sta.*, 79-80: 491-534. 1913.

Air-drying of soil very markedly increases the amount of water soluble substances especially the inorganic soil constituents<sup>10</sup> (table 99). Achromeiko<sup>11</sup> found that drying of soil increases the water soluble  $P_2O_5$ , organic matter and mineral soil constituents. When the soil is moistened again, there is a decrease in these and an increase in  $NO_3^-$ . When the dried soils are kept under sterile conditions, there is no change in the water soluble organic matter, but the soluble  $P_2O_5$  gradually decreases.

According to Bigini,<sup>12</sup> drying of soil is less injurious to nitrification than to other soil bacteriological processes. The addition of green manure to soil brings about a considerable increase in bacteriological activities even in very dry soils, with only 4.6 per cent moisture. Cultivation of the soil has in itself a stimulating effect upon the bacterial activities; this is, however, negligible in comparison with the effect of

TABLE 99  
*Increase in total soluble salts due to drying*

SOIL LAYER	INCREASE DUE TO OVEN-DRYING OF WET SOIL	INCREASE DUE TO AIR-DRYING OF WET SOIL	INCREASE DUE TO OVEN-DRYING OF AIR-DRY SOIL
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Surface, 0 to 8 inches.....	60	23	50
Subsurface, 12 to 20 inches.....	62	74	59
Subsoil, 20 to 40 inches.....	200	94	104

drying. Proper aeration supplies the oxygen necessary for bacterial activities, and brings about more fundamental changes influencing these activities. This is particularly true when cultivation of soil is accompanied by air-drying.

*Influence of caustic lime upon soil processes.* Caustic lime (about 0.5 per cent) is recognized as an antiseptic. When applied to soil, even in the presence of large quantities of  $CaCO_3$ ,  $CaO$  will disturb or even destroy the state of equilibrium normally existing between the

<sup>10</sup> Kelley, W. P. Hawaii Agr. Exp. Sta. Bul. 37, 1915; Gustafson, A. F. Soil Sci., 13: 173-213. 1922.

<sup>11</sup> Achromeiko, A. Ztschr. Pflanzen. Düng. Bodenk., 11A: 65-89. 1928; see also Khalil, F. Centrbl. Bakt. II, 79: 93-107. 1929; Salewski, W. and Kucharkowa, A. Pedology, 23: 94-112. 1928; Burgess, R. Centrbl. Bakt. II, 78: 497-507. 1929.

<sup>12</sup> Bigini, C. Staz. sper. agrar. ital., 59: 114-129. 1926 (C. A. 22: 1643. 1928); see also Rudakov, K. I. Bakteriologo-Agron. Sta. Moscow., 24: 15-51. 1926.

micro-flora and micro-fauna of the soil.<sup>13</sup> The action of CaO is intermediate between the action of antiseptics and changes induced by high temperatures. It seems to consist in bringing about a greater decomposition of the organic nitrogen constituents of the soil. The numbers of bacteria are at first depressed but are later observed to increase.<sup>14</sup> Many bacteria and the larger protozoa are destroyed. The numbers of bacteria remain depressed until the excess of the calcium oxide is transformed into carbonate when active bacterial multiplication takes place. The action of lime depends, of course, upon the character of the soil, each soil neutralizing a definite amount before the phenomenon of partial sterilization becomes evident; when there is no further absorption of lime, the free alkali begins to function as a disinfectant. This is the reason why  $\text{CaCO}_3$  does not produce the same effect.

*Partial sterilization of soil.* Russell and Hutchinson<sup>15</sup> demonstrated that partial sterilization of soil (heating to 60°C. or treatment with volatile antiseptics) increased the rate of oxidation, as well as the numbers of bacteria. Similar observations for bacterial numbers were made previously (1901) by Hiltner and Störmer,<sup>16</sup> who treated the soil with  $\text{CS}_2$ . Russell and Hutchinson also found that the high numbers in partially sterilized soil were increased if a little fresh untreated soil was added; later, however, the bacterial numbers in the reinoculated soil fell.

Partial sterilization of soil can be accomplished by means of heat or by treating the soil with various antiseptics. By the use of steam or dry heat, the soil can be partially or completely sterilized, depending on the temperature and length of treatment. To sterilize a soil completely, it must be subjected to steam pressure for two hours at 15 pounds, or for one hour, on seven consecutive days, in flowing steam. Temperatures less than 100°C. only destroy certain groups of microorganisms; at 60° to 65°, for example, living protozoa, particularly the large forms, fungus mycelium and spores,<sup>17</sup> as well as vegetative cells of various bacteria, are destroyed. To partially sterilize a soil is to treat it in such a manner as to destroy certain groups of organisms and leave others uninjured. Soil partially or completely sterilized by heat be-

<sup>13</sup> Hutchinson, H. B. Jour. Agr. Sci., 5: 320. 1913; 6: 302. 1914.

<sup>14</sup> Fischer, H. Landw. Vers. Sta., 70: 335-342. 1909; Miller, F. Ztschr. Gärungsphysiol., 4: 194-206. 1914.

<sup>15</sup> Russell and Hutchinson, 1909-1913 (p. 298).

<sup>16</sup> Hiltner and Störmer, 1903 (p. 12).

<sup>17</sup> Thom and Ayers, Jour. Agr. Res., 6: 153-166. 1916.

comes a favorable medium for the growth of certain groups of fungi and bacteria. A large number of antiseptics bring about true partial sterilization with the following results:<sup>18</sup>

- a. An initial decrease in the number of bacteria followed by a large sustained rise.
- b. The destruction of the protozoa and nitrifying organisms.
- c. An initial increase of ammonia followed by a considerable increase in the rate of ammonia formation and consequently of soil productivity.
- d. No increase in the dose of the antiseptic causes any change in the results obtained, once true partial sterilization has set in.
- e. The complete or almost complete destruction of the soil fungi,<sup>19</sup> of soil nematodes and other soil infesting worms and insects.<sup>20</sup>

True partial sterilization has been obtained only with the easily volatile or removable antiseptics. Non-removable substances have a

TABLE 100  
*Influence of toluol upon soil microorganisms and their activities*<sup>18</sup>

TOLUOL ADDED PER KILOGRAM OF DRY SOIL	BACTERIA PRESENT, MILLIONS PER GRAM OF DRY SOIL			AMMONIA + NITRATE, PARTS PER MILLION OF DRY SOIL		EFFECT ON PROTOZOA
	Start	34 days	51 days	Start	30 days	
Untreated = 0 gram.....	22.0	16.0	18.0	24.0	43.0	CAF*
M/200 = 0.46 gram.....	16.0	22.5	18.5	26.5	50.0	CF
M/100 = 0.92 gram.....	8.5	76.0	92.0	28.5	56.0	F
M/50 = 1.84 grams.....	7.0	87.0	94.0	29.0	63.0	(F)
M/10 = 9.2 grams.....	8.0	95.0	87.0	29.5	65.0	0
M/5 = 18.4 grams.....	8.0	77.0	90.0	29.5	66.0	0
M = 92.0 grams.....	7.0	90.0	86.0	30.0	66.0	0

\* C = ciliates; A = amoebae; F = flagellates.

lasting effect upon the flora, stimulating only a few species, without bringing about, however, a considerable gain in ammonia or nitrate. Larger doses of these chemicals may even suppress microbiological activities. The influence of the concentration of the antiseptic is given in table 100.

Among the volatile antiseptics, toluol, carbon bisulfide and chloroform have been commonly employed. Concentrations of 1 to 4 per

<sup>18</sup> Buddin, W. Jour. Agr. Sci., 6: 417-451. 1914; Russell, E. J. Intern. Inst. Agr., Bur. Agr. Int. Pl. Dis., 8: No. 5. 1917.

<sup>19</sup> Waksman and Starkey, 1923 (p. 717).

<sup>20</sup> Russell, E. J. Jour. Roy. Hort. Soc., 55: 236-246. 1920.

cent of the disinfectant are allowed to act upon the soil for 12 to 48 hours; the soil is then aerated so that the disinfectant may evaporate. Among the non-volatile antiseptics, it is sufficient to mention phenol, cresol (M/10), cresylic acid, naphthalene, various metallic salts, like arsenic compounds. Some of these, like the phenols, are decomposed in course of time by various bacteria; others, like the arsenic oxide, may persist for some time in the soil. The intensity of partial sterilization shades off gradually from the powerful non-volatile antiseptics, through cresol (M/50) and formaldehyde, to the more or less potent volatile antiseptics, until finally a hardly noticeable effect is obtained, as in the case of merely spreading out the soil in a thin layer.

*The use of heat as an agent of partial sterilization.* Franke<sup>21</sup> observed that heating of soil increases the solubility of the mineral constituents and of the organic matter in the soil, as well as of soil productivity. Krüger and Schneidewind<sup>22</sup> also suggested that the favorable effect of heat is due to the increase in the solubility of soil minerals, as shown in the following summary:

*Yield of mustard, in grams per pot*

FERTILIZATION	NO MANURE	NaNO <sub>3</sub>	COMPLETE MINERAL FERTILIZER	NaNO <sub>3</sub> + COMPLETE MINERAL FERTILIZER
Untreated soil.....	17.3	17.5	33.7	50.9
Heated soil.....	33.2	36.5	46.9	62.4

It was later found that heating of soil brings about a decided change in the microbial population. On subsequent remoistening of the soil, the greatest increase in the numbers of microorganisms is shown by the non-spore forming bacteria; the actinomyces increase only slowly, while the fungi and protozoa destroyed by the treatment reappear only later. The fungi once introduced make a very rapid growth on soil sterilized by heat.<sup>23</sup> Sterilization by heat results also in the destruction of the nitrifying bacteria; it brings about a decided increase in the amount of ammonia accumulated in the soil, due to a greater decomposition of the soil organic matter by the microorganisms. This accounts for the

<sup>21</sup> Franke, B. Ber. deut. bot. Gesell. Generalsammlungsh., 6: 87-97. 1888.

<sup>22</sup> Krüger, W. and Schneidewind, W. Landw. Jahrb., 28: 217-252. 1899.

<sup>23</sup> This phenomenon is readily observed also in field soils, which became subject to considerable heat for one reason or another: Tokugawa, Y., and Emoto, Y. Japanese Jour. Bot., 2: 175-188. 1924.

increased fertility of the soil and beneficial influence upon plant growth, which results from the steaming of the soil.<sup>24</sup>

Fischer<sup>25</sup> observed that when a soil is sterilized by means of steam and reinoculated with bacteria, a great increase in biological activities results; this can be determined by the increase in numbers and by the production of carbon dioxide. After some time (three weeks), the bacterial numbers still remain at a high level while the production of carbon dioxide rapidly diminishes. Fischer suggested, therefore, that after a period of decided activities the bacteria become rather inactive although they still show increased numbers; they go into "resting" forms, which possess a low respiratory power, but are capable of developing on the plate into colonies. The rapid increase of bacteria was not found to be accompanied by a similar increase of the fungi. The increase in bacterial activities was considered to be a result of the decomposition of the bodies of microorganisms in soil. The increased numbers of bacteria are believed to bring about an increase in the decomposition of the soil constituents, both organic and inorganic.

Heating of peat soil, at 100° for fifteen minutes, was found<sup>26</sup> to stimulate greatly the biological activities, when the evolution of carbon dioxide (from 200 grams of soil) was used as an index of these activities. This favorable influence was found to be due not to the destruction of toxins or protozoa, but to a chemical modification of the peat. A stimulative effect of steam treatment of soil upon the numbers of bacteria has also been observed.<sup>27</sup> The results of heating (65°C. for one hour) of a soil upon the numbers of fungi and bacteria, evolution of carbon dioxide and accumulation of available nitrogen (ammonia + nitrates) are shown in figure 71. Heating a soil even to low temperatures seems to improve it as a medium for bacterial growth. It is interesting to mention, in this connection, the changes brought about by heat in the physical and chemical conditions of the soil. While treatment of soil at temperatures lower than 100°C. render the soil more fertile, temperatures higher than 100° render it less fertile.<sup>28</sup> The lower temperatures bring about an increase in the soluble organic matter;<sup>29</sup> treatment of soil at

<sup>24</sup> Elveden, V. Jour. Agr. Sci., 11: 197-210. 1921.

<sup>25</sup> Fischer, H. Centrbl. Bakt. II, 22: 671-675. 1909.

<sup>26</sup> Demolon, A. and Boischot, P. Compt. Rend. Acad. Sci., 177: 282-284. 1923; Lundblad, K. Svensk. Mosskult. Tidskr., 44: 273-305. 1930.

<sup>27</sup> Osmun, A. V. Mass. Agr. Exp. Sta. Rpt. 1905, 146-148.

<sup>28</sup> Pickering, S. U. Jour. Agr. Sci., 3: 32-54, 258-276. 1910.

<sup>29</sup> Schreiner, O. and Lathrop, E. C. U. S. Dept. Agr. Bur. Soils. Bul. 89. 1912; Jour. Amer. Chem. Soc., 34: 1142-1159. 1912.

higher temperatures (especially above 100°C.) results in the formation of substances toxic to the growth of higher plants: among those, guanine, arginine, dihydroxystearic acid have been recorded. There is also an increase in acidity, or rather ability of soil to neutralize bases.<sup>30</sup> The toxic substances are unstable and gradually disappear in the course of time, if the soil is kept moist and aerated.

The increase in soluble organic matter, as a result of heating, varies directly with the temperature to which the soil is subjected, particularly in the case of carbohydrates. It has also been observed that heating of soil brings about a greater solubility of the phosphorus and nitrogen

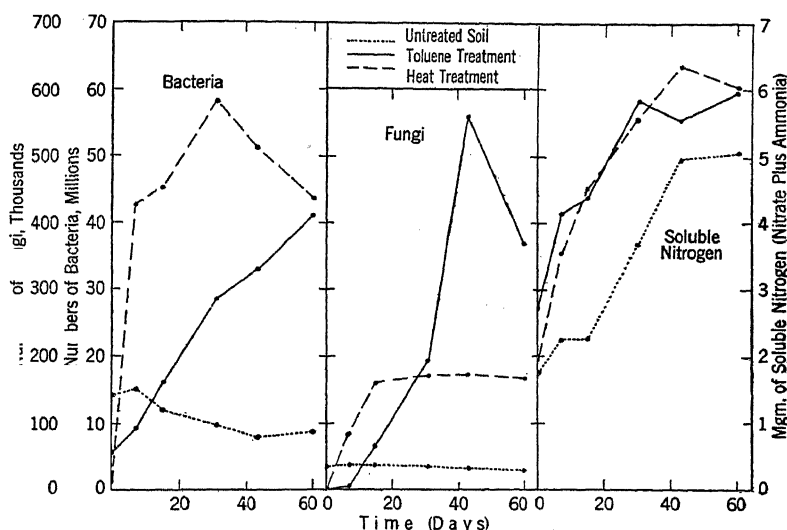


FIG. 71. Influence of toluol and heat upon the numbers and activities of micro-organisms in soil (after Waksman and Starkey).

compounds in the soil.<sup>31</sup> Similar results were obtained by a number of other investigators.<sup>32</sup> Bouyoucos<sup>33</sup> and Wilson<sup>34</sup> found an increase

<sup>30</sup> Robinson, R. H. *Soil Sci.*, 9: 151-157. 1920.

<sup>31</sup> Liebscher, G. *Deut. Landw. Presse.*, 20: 975-976. 1893; Krüger, W. and Schneidewind, W. *Landw. Jahrb.*, 28: 217-252. 1899.

<sup>32</sup> Whitney, M. and Cameron, F. K. *U. S. Dept. Agr., Bur. Soils, Bul.* 22, 1-71. 1903; Lyon, T. L. and Bizzell, J. A. *N. Y. (Cornell) Agr. Exp. Sta. Bul.* 275, 1910; *Bul.* 326, 1913; Seaver, J. F. and Clark, F. D. *Mycologia*, 2: 109-124. 1910; *Biochem. Bul.* 1: 413-427. 1912; Gustafson, 1922 (p. 720).

<sup>33</sup> Bouyoucos, G. *Mich. Agr. Exp. Sta. Tech. Bul.* 24. 1915.

<sup>34</sup> Wilson, A. *Proc. Roy. Dublin Soc. N. S.*, 38: 513. 1915.



in the concentration of the soil solution as a result of heating of soil, using the freezing point method. This increase is greater in soils rich in organic matter than in mineral soils. Heat also effects a flocculation of the soil colloids, thus changing the physical condition of the soil.

The increase in the soluble matter and the changes in the microbiological population of the soil brought about by heating result in an increase in the numbers of bacteria; these in their turn decompose more organic matter, which results in a greater liberation of available nitrogen. This favors the growth of plants.

Partial sterilization of soil by heat is widely practiced in greenhouse cultivation of plants. Not only are the pests and various diseases eliminated from the seed bed, but increased fertility and vigor of plants result. The soil is heated to just below the boiling point of water and kept at that temperature for 15 to 40 minutes, depending on soil type, heavier soils requiring longer periods of time. The heat is obtained either by passing steam through a heap of soil or by baking the soil in an oven or other suitable apparatus. The soil should not be too wet or too dry and the heat must be uniformly distributed.<sup>35</sup>

*Influence of volatile antiseptics upon bacterial activities in the soil.* Volatile antiseptics, especially carbon bisulfide, were first applied to soils and plants for the destruction of insect and fungus pests. As far back as 1870 the observation was made that this disinfectant has a stimulating effect upon plant growth. Girard<sup>36</sup> used CS<sub>2</sub> to clear a piece of sugar-beet land badly infested with nematodes and observed marked increases in the succeeding crops as a result of the treatment. In 1894 Oberlin<sup>37</sup> applied CS<sub>2</sub> for the destruction of *Phylloxera* and noticed that the productiveness of the soil was greatly increased. He suggested, therefore, that soil sickness can be corrected by the application of the disinfectant and ascribed this favorable influence to the destruction of injurious microorganisms. This effect was also explained by the direct stimulation of young plants.<sup>38</sup>

<sup>35</sup> Russell, E. J. and Petherbridge, F. R. Jour. Agr. Sci., 5: 248-287. 1913; Mann, H. H., Joshi, N. V. and Kanitkar, N. V. Mem. Dept. Agr. India, Chem. Ser., 2: 141-192. 1912; Kelley, W. P. and McGeorge, W. Hawaii Agr. Exp. Sta. Bul. 30. 1913; Demolon, A. Intern. Rev. Sci. Pract. Agr., 3: 431. 1924 (Chem. Abstr., 20: 1878); Bewley, W. F. Jour. Min. Agr., 36: 623-633. 1929.

<sup>36</sup> Girard, A. Bul Soc. Nat. Agr. France, 54: 356-363. 1894.

<sup>37</sup> Oberlin. Bodenmüdigkeit und Schwefelkohlenstoff. Mainz. 1894.

<sup>38</sup> Koch, A. Arb. deut. landw. Gesell. H. 40. 1899; Egorov, M. A. Zhur. Opit. Agron., 9: 34-95. 1908.

Nobbe and Richter<sup>39</sup> obtained a definite increase in crop yield by treatment of soil with ether, chloroform or benzol. It was soon observed that the stimulating effect applies to all soils and all plants.<sup>40</sup> The antiseptics, like carbon bisulfide, carbon tetrachloride, toluol, benzol, phenol, cresol, are in themselves directly poisonous to plants when added to water cultures. They were found to exert a decided stimulating effect when added to the soil some time before the crop is planted. This was attributed<sup>41</sup> to a changed bacterial population. A bacterial flora of  $9\frac{1}{2}$  millions per gram, as determined by the plate method, was depressed by the addition of the disinfectant (fig. 72):

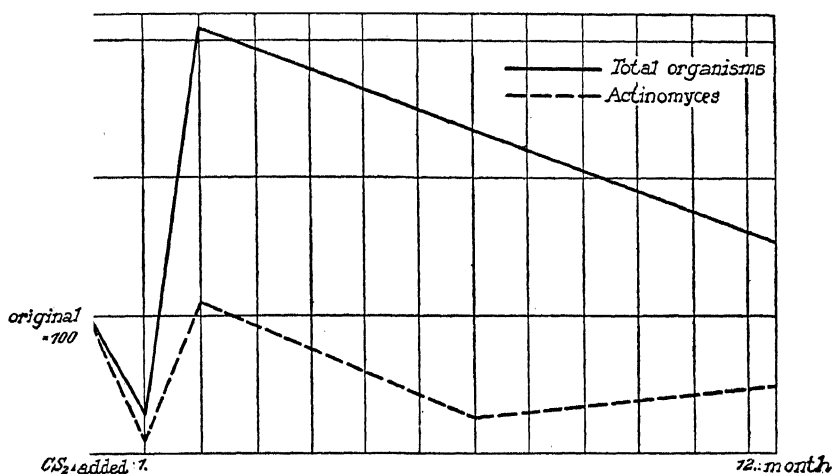


FIG. 72. Influence of  $\text{CS}_2$  upon the numbers of bacteria and actinomycetes in soil (from Hiltner and Störmer).

the numbers soon increased, however, reaching, within a month after the evaporation of the disinfectant, 50 millions; the numbers then slowly fell but remained above the original figure. The normal flora of the soil consisted of 75 per cent non-liquefying bacteria, 20 per cent actinomycetes and 5 per cent gelatin liquefying forms. As a result of treatment with carbon bisulfide, the non-liquefying organisms were greatly increased,

<sup>39</sup> Nobbe, F. and Richter, L. *Landw. Vers. Sta.*, 60: 433-448. 1904.

<sup>40</sup> Störmer, K. *Jahresb. ver. angew. Bot.* for 1907, 5: 113-131. 1908; *Centrbl. Bakt. II*, 20: 282-288. 1908.

<sup>41</sup> Hiltner and Störmer, 1903 (p. 12); Hiltner, L. *Jahresb. Angew. Bot.*, 5: 200-222. 1907; *Centrbl. Bakt II*, 38: 228. 1912.

while the actinomyces were reduced and did not return to the original proportion for at least two years. The denitrifying bacteria were completely destroyed, while the pectin-fermenting organisms were reduced. As a result of these studies, Hiltner and Störmer came to the following conclusions:

1. Destroying the existing bacterial equilibrium in the soil, carbon bisulfide opens the way for an entirely new bacterial development. This is achieved through the unequal retardation in the growth of the different groups of bacteria. Hence certain groups become disproportionately prominent, while others are almost entirely suppressed.

2. The rapid increase in the numbers of the bacteria is followed by a more intense transformation of plant food substances. Decomposition and fixation processes result in an accumulation of readily available nitrogen compounds utilized by the crops. Hence the action of carbon bisulfide is in the nature of nitrogen action.

3. The initial suppression of the nitrifying species becomes of advantage in that the nitrogen compounds, simplified by other species, are prevented from being rapidly changed into nitrates and being leached out of the soil.

4. The more or less permanent suppression of the denitrifying organisms must be regarded as an additional factor favoring plant growth.<sup>42</sup>

Van Suchtelen<sup>43</sup> found that CS<sub>2</sub> stimulates the decomposition of organic matter, as indicated by the CO<sub>2</sub> production; 12 kgm. of soil + 6 grams glucose gave 7,215 milligrams of CO<sub>2</sub> in nineteen days when treated with 170 grams of CS<sub>2</sub>, while the untreated soil gave 5,991 milligrams. Soil treated with heat or volatile antiseptics has a much greater oxidizing power, as indicated by the oxygen absorption by the soil.<sup>44</sup>

Störmer suggested that the disinfectants kill the larger soil organisms, such as worms, insects, fungi, algae, protozoa; these are then decomposed by the surviving bacteria with the formation of ammonia. Bacterial development and ammonia accumulation are a result of this decomposition. The total increase in ammonia nitrogen over the untreated soil is not more than 3 to 4 mgm. of nitrogen per 100 grams of soil; this quantity can be readily derived from the decomposed organisms. Garden soils may be very rich in nematodes which often do great damage to the crop.<sup>45</sup> These nematodes are destroyed by the disinfectant.

<sup>42</sup> The observations of Hiltner and Störmer were confirmed by Moritz, J. and Sherpe, R. *Arb. K. Gesundheitsamt. Biol. Abt.*, **4**: 123-156. 1904; also *Arb. K. Biol. Anst. Land. Forstw.*, **7**: 353-425. 1909; *Centrbl. Bakt.* **II**, **13**: 573.

<sup>43</sup> Van Suchtelen, 1910 (p. 700).

<sup>44</sup> Darbishire, F. V. and Russell, E. J. *Jour. Agr. Sci.*, **2**: 305-326. 1908.

<sup>45</sup> Emmerich, R. W., Graf zu Leiningen and Loew, O. *Centrbl. Bakt.* **II**, **29**: 668. 1911; **31**: 466-477. 1911.

According to Stoklasa,<sup>46</sup> the increase in soil fertility due to treatment with CS<sub>2</sub>, chloroform, benzol, or ether is due to the destruction of a definite number of soil microorganisms; the surviving bacteria readily break down the dead organisms, liberating phosphate and other ions which now become available for plant growth. It is to be noted that among the organisms which develop in great abundance in partially sterilized soils *Clostridium pastorianum*, the anaerobic nitrogen-fixing organism, occupies a prominent place, occurring as 100,000 or more per gram of soil.<sup>47</sup>

It thus became evident that the treatment of soil with antiseptics is equivalent to nitrogen fertilization. It was suggested<sup>48</sup> that partial sterilization of soil renders a number of undecomposed plant residues,

TABLE 101  
*Numbers of bacteria in untreated and partially sterilized soils*<sup>55</sup>

	AT START	END OF FIRST PERIOD	END OF SECOND PERIOD	END OF THIRD PERIOD	END OF FOURTH PERIOD
		16 days	30 days	74 days	
Untreated.....	27 millions	10 millions	10 millions	45 millions	
CS <sub>2</sub> .....	2 millions	17 millions	53 millions	121 millions	
		15 days	110 days	170 days	200 days
Untreated.....	13 millions	9 millions	4 millions	9 millions	12 millions
65°C.....	13 millions	21 millions	37 millions	45 millions	60 millions
		40 days	100 days	160 days	500 days
Untreated.....	11 millions	16 millions	9 millions	13 millions	6 millions
Toluol.....	2 millions	43 millions	41 millions	43 millions	18 millions

such as pectins and pentosans, more soluble; these are used as sources of energy by the nitrogen-fixing bacteria; the subsequently more intense transformation of the bacterial proteins and of other nitrogenous organic substances into amino- and ammonium compounds places an abundant and uniform supply of soluble nitrogen compounds at the disposal of the plant.

The original idea of Koch<sup>49</sup> that increased crop growth due to the

<sup>46</sup> Stoklasa, 1911 (p. 570).

<sup>47</sup> Truffaut, G. and Bezssonoff, N. *Compt. Rend. Acad. Sci.*, 170: 1278-9. 1920; 171: 268-270. 1920; 172: 1319-1323. 1921.

<sup>48</sup> Heinze, B. *Centrbl. Bakt. II*, 16: 329-357. 1906; 18: 56, 246, 462, 624, 790. 1907; *Landw. Jahrb.*, 36: 418. 1907.

<sup>49</sup> Koch, 1899 (p. 726).

application of the disinfectant is a result of a direct stimulation of the plant by traces of the disinfectant or its decomposition products

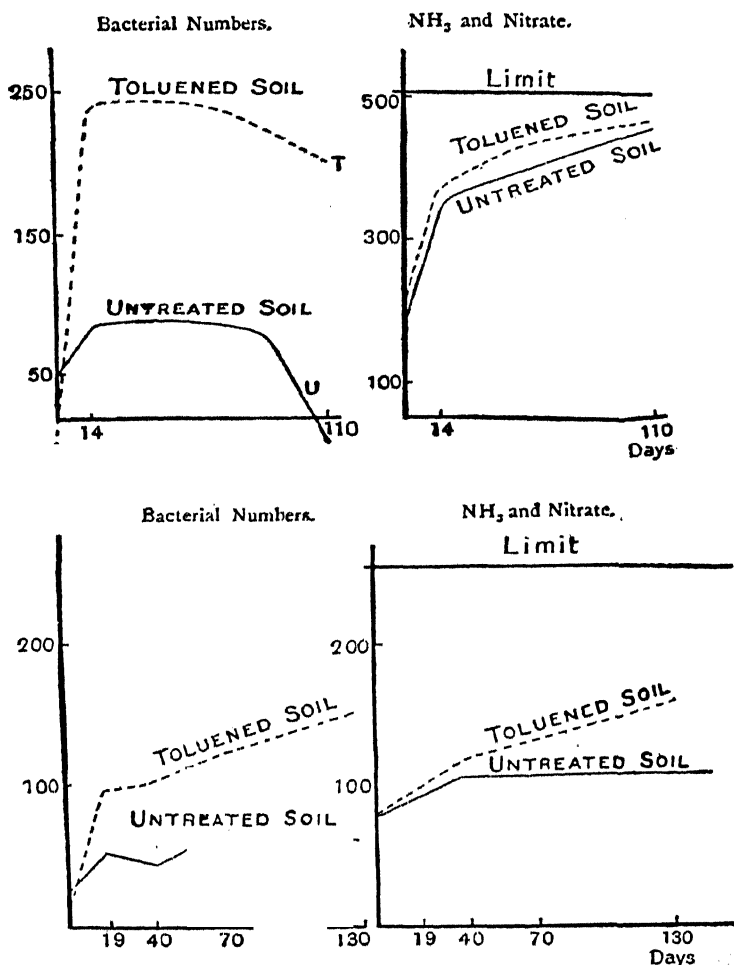


FIG. 73. Influence of toluol upon numbers of bacteria and available nitrogen (ammonia and nitrate) formed in the soil; upper figures from a soil in which small amounts of available nitrogen were initially present; lower figures from a soil in which large amounts of available nitrogen were initially present (after Russell).

found various adherents. Fred<sup>50</sup> observed that proper concentrations of ether,  $\text{CS}_2$  and  $\text{CuSO}_4$  have a stimulative effect upon the growth of

<sup>50</sup> Fred, E. B. Centrbl. Bakt. II, 31: 185-245 1912.

lower microorganisms; even nitrification, which was at first decreased, was later stimulated.  $\text{CS}_2$ , in dilute solutions, stimulates growth of plants, including that of fungi.<sup>51</sup> According to Hüne,<sup>52</sup> small doses of poisons may be directly stimulative to bacterial development.

The influence of antiseptics upon the development of microorganisms in the soil, namely those that can be determined by the plate method, is brought out in table 101. A large increase in soluble nitrogen, as a result of treatment with  $\text{CS}_2$ , takes place both in the inoculated and uninoculated soils. The ammonia content of the soil follows the curve of bacterial growth and later gives rise to nitrates.  $\text{CS}_2$  does not act alike on all soils and toward all crops.

Russell and associates (fig. 73) attempted to correlate the destruction of protozoa following partial sterilization with the increase in the numbers of bacteria and their activities and subsequently soil fertility. They suggested that the protozoa are responsible for keeping down bacterial numbers in an untreated soil and, therefore, affect adversely the production of plant food. The partial sterilization of the soil results in the destruction of protozoa, thus removing the agent injurious to normal bacterial development.

The various theories and hypotheses that have been proposed in explanation of the favorable influence of partial sterilization of soil upon its fertility can be summarized as follows:

1. *Direct stimulation theory.* Plant roots and microorganisms may be stimulated directly by small quantities of antiseptics;<sup>53</sup> it has been suggested<sup>54</sup> that the latter are used directly as nutrients by microorganisms.

2. *Indirect stimulation of bacteria.* The organic matter in the soil may be modified in such a manner, as a result of partial sterilization, as to make it more available for bacterial action; this may be due either to the removal of the fats, to greater solubility of carbohydrates, nitrogen compounds, or phosphates; to the killing of worms, nematodes, protozoa, algae, fungi, which are then decomposed by the bacteria; or to all these combined.

3. *Microbiological balance or equilibrium.* Partial sterilization produces a change in the balance between the bacterial flora and the other groups of organisms, such as the fungi and actinomyces.

4. *The protozoa* are responsible for the limitation of bacteria in the soil; their removal by partial sterilization leads to increased bacterial development, greater decomposition of organic matter and, therefore, improved soil fertility.

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<sup>51</sup> Oldenbusch, C. Bull. Torrey Bot. Club., 49: 375-390. 1922.

<sup>52</sup> Hüne, Dr. Centrbl. Bakt. I, Orig., 4: 135-140. 1907.

<sup>53</sup> Maassen, A. and Behn, H. Mitt. K. Biol. Anst. Land. u. Forstwirt., 12: 285-338. 1924.

<sup>54</sup> Matthews, A. Jour. Agr. Sci., 14: 1-57. 1924; Jacobs, S. E. Ann. Appl. Biol., 18: 98-136. 1931.

5. *Toxin theory.* The soil is believed to contain toxins of biological origin. Partial sterilization of soil leads to their destruction, hence to improved fertility.

6. *Destruction of fungi and bacteria which are causative agents of plant diseases.* The repeated growth, year after year, of the same crop leads to an accumulation of fungi and insects injurious to the particular crop. Partial sterilization of soil brings about the destruction of these pests.

7. *Increased nitrogen-fixation.* Partial sterilization of soil is believed to render a greater amount of energy available to the nitrogen-fixing bacteria. Koch, stated, however, that nitrogen fixation by bacteria is decreased by partial sterilization.

*Protozoan theory.* The "protozoan theory of soil fertility" advanced by Russell and Hutchinson<sup>55</sup> has met with severe criticisms. According to this theory, the number of bacteria found in soil, at any given time, is not merely a function of environmental soil conditions, but depends on the interrelationship between the bacteria and the protozoa; partial sterilization does not bring about an improvement in the bacterial flora but makes the soil a better medium for the growth of bacteria, by eliminating the detrimental factor.

It was suggested<sup>56</sup> that the destruction of spores of disease producing fungi and bacteria has more to do with the final increase in productivity of heated soils than either the destruction of bacteria-loving protozoa or the increase in soluble plant food. The increase in ammonia formation in partially sterilized soils was believed to be due to fungi.<sup>57</sup> The fact that fungi grow readily in soils subjected to dry or moist heat<sup>58</sup> tends to add further weight to this idea. The abundant growth of fungi in soils treated with disinfectants, after a certain period has elapsed, is also quite marked. Figures 74 and 75 show that the inoculation of soil with protozoa may lead to a decided depression in the numbers of bacteria;<sup>59</sup> however, so far no definite proof has been submitted that the introduction of protozoa actually depresses in the soil biochemical processes important to soil fertility; the meagre results available seem to point to the contrary, as shown elsewhere (p. 322).

*Agricere and Bacteriotoxin theories.* The results of Schreiner,

<sup>55</sup> Russell and Hutchinson, 1909-1912 (p. 298).

<sup>56</sup> Bolley, H. L. *Science*, **33**: 229-234. 1911; also **32**: 529-541. 1910; **38**: 48-50, 249-259. 1913; N. D. Agr. Exp. Sta. Bul. 107. 1913; Jachscheski, A. Khoziastvo, 1912, 1103-1108 (Intern. Inst. Agr. Bul. Bur. Agr. Inst. Pl. Dis., **3**: 2528).

<sup>57</sup> Kopeloff, N. and Coleman, D. *Soil Sci.*, **3**: 197-269. 1917; Skinner, C. E. *Soil Sci.*, **24**: 149-162. 1927.

<sup>58</sup> Seaver and Clark, 1912 (p. 725).

<sup>59</sup> Cutler, 1922 (p. 29).

Shorey and their associates seemed to indicate that the soil organic matter contains certain organic complexes which are distinctly injurious to crop growth. Livingston<sup>60</sup> regarded the general hypothesis that

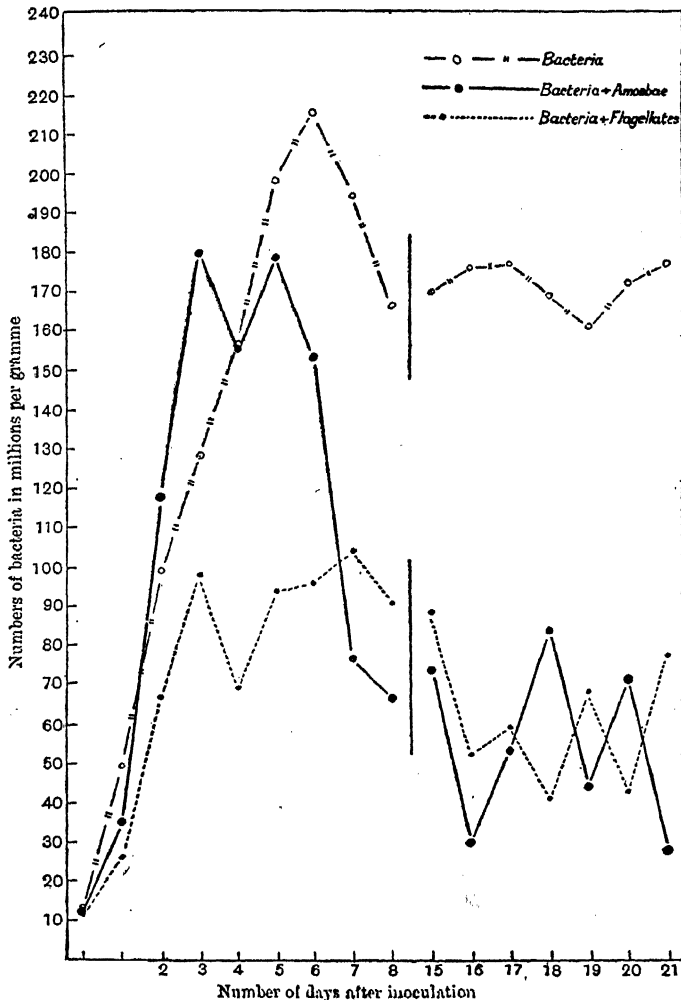


FIG. 74. Action of protozoa upon the development of bacteria (from Cutler).

unproductiveness of agricultural soils is due frequently to soil toxins as well established and generally accepted.

<sup>60</sup> Livingston, B. E. Palladin's Plant Physiology. 1st Ed. 1918, p. 93.



According to Greig-Smith,<sup>61</sup> no one phenomenon can explain the cause of the enhanced fertility of soils treated with volatile antiseptics. Bacteria growing in any culture medium produce injurious or toxic products, which check and inhibit their further growth. These toxins may be

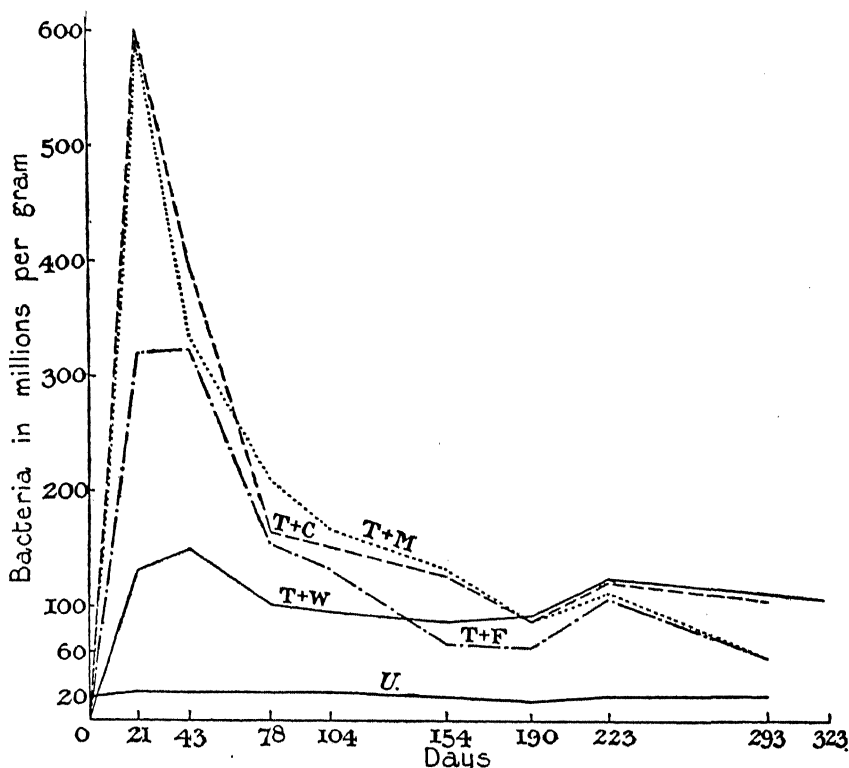


FIG. 75. Influence of protozoa upon the development of bacteria: U = uninoculated; T + W = toluol treated soil plus distilled water; T + F = toluened soil plus hay-infusion, filtered through a Berkefeld filter; T + C = toluened soil plus ciliates in hay-infusion; T + M = toluened soil plus mixed culture of protozoa and bacteria in hay-infusion (from Goodey).

in the nature of lysins, acids, accumulated by-products, etc. Soil extract filtered through porcelain had a destructive influence upon *Bact. prodigiosum*, while the same extract heated and treated by sunlight or allowed to stand in aqueous solution stimulated bacterial growth.

<sup>61</sup> Greig-Smith, R. Centrbl. Bakt. I., 34: 224-226. 1912; Proc. Linn. Soc. N. S. Wales, 36: 679-699. 1911.

Greig-Smith considered that, in addition to toxic substances, the soil contains a mixture of fatty substances (*agricere*) derived from plant material. These fats are not readily acted upon by microorganisms and finally cover and impregnate the residual organic matter. Volatile antiseptics, being fat solvents, dissolve the *agricere*, which is either carried toward the surface of the soil or is segregated upon the points and angles of the individual soil particles. This was believed to be the cause of the favorable influence of the action of antiseptics upon bacterial growth and activities. When the soil fats are removed or segregated, the soluble matter diffuses out more readily from the soil particles and serves as a source of food for microorganisms.<sup>62</sup>

Treatment with volatile antiseptics was found to induce an increased growth of bacteria in soils, in which the protozoa have been destroyed by moist or dry heat at 65° to 75°C. It was suggested, therefore, that two factors influence bacterial activities in the soil, one of which is a bacteriotoxin and which is destroyed by heat, and the other a fat or wax, which is dissolved by volatile antiseptics. According to Hutchinson,<sup>63</sup> the formation of toxic substances depends upon the existence of anaerobic conditions due to water logging; when organic matter is decomposed under these conditions, waxes and slimes are formed which coat the soil particle tending to block up its pores, thus interfering with aeration and drainage, and protecting the organic particles from the further action of the bacteria. Greig-Smith admitted that protozoa may play a part in checking the multiplication of bacteria in soil, but they were not believed to be alone responsible for this limitation. These results on the formation of a bacteriotoxin in the soil were not confirmed by other investigators.<sup>64</sup> However, certain metabolic products are formed by at least some soil microorganisms, which are either toxic to themselves or to other organisms. The removal, destruction or modification of these products may bring about an increased activity of the microorganisms concerned.

Treatment of soil by heat or antiseptics results in a number of very complex processes, which cannot be explained by a protozoan theory, toxin theory, presence of soil waxes, etc., although all of these may play a certain part in limiting bacterial development. A series of changes

<sup>62</sup> Greig-Smith, R. Proc. Linn. Soc. N. S. Wales, **35**: 808-822. 1910; **36**: 679-699. 1911; **37**: 238-243, 655-672. 1912; **38**: 725-746. 1913; **39**: 839-850: 1914; **40**: 631-645, 724-733. 1915; **42**: 162-166. 1917; **43**: 142-190. 1918.

<sup>63</sup> Hutchinson, C. M. Agr. Jour. India, **21**: 125-133. 1926.

<sup>64</sup> Hutchinson, H. B. and Thaysen, A. C. Jour. Agr. Sci., **9**: 43-62. 1918.

are brought about which lead to an increase in the soluble soil organic matter, and to a modification of the concentration of the soil solution and of the soil reaction. The physical condition (permeability, capillarity, cohesion, surface tension) of the soil is also modified by the treatment, particularly the colloidal properties of the soil.<sup>65</sup> Quantities of toluol and CS<sub>2</sub>, insufficient to modify the number and types of protozoa in soil, were found<sup>66</sup> to exert a stimulative effect upon bacterial activities.

*Destruction of selective groups of organisms.* Different soil organisms are not affected alike by disinfectants.<sup>67</sup> Some are injured but not completely destroyed. The degree of injury also depends upon the concentration and length of action of the disinfectant, on the soil moisture and aeration conditions. The gelatin liquefying bacteria, mostly spore-formers, are not affected at all or to only a very limited extent, while the actinomyces may be considerably reduced; the same is true of other aerobic, especially non-spore-forming, bacteria. When the disinfectant is evaporated, there is a rapid increase in the non-liquefying forms; the activity of the actinomyces and gelatin liquefying bacteria lags very much behind. After the maximum of bacterial activities is attained, a gradual decrease follows and within about a year the flora of the treated soil has reached the level of the untreated soil.

The most active bacteria, after partial sterilization, were found<sup>68</sup> to be *Bac. butyricus*, *Bac. mycoides*, *Bac. megatherium*, *Bac. arborescens*, *Microc. ochraceus luteus* and, to a less extent, *Bact. fluorescens liquefaciens*. Various other investigators found<sup>69</sup> actinomyces to be decidedly injured by treatment with CS<sub>2</sub>; the *Bac. mycoides* group was less affected, the spore formers being especially resistant. Legume bacteria (peas) and denitrifying bacteria (*B. stutzeri*) are rapidly killed, within two and a half hours; *Bact. coli*, *Bact. prodigiosum*, *Bact. vulgare*, *Mic. ureae* survive four hours. Staphylococci are very resistant, surviving forty-eight hours. *Az. chroococcum* survives in moist soil impregnated with fumes of CS<sub>2</sub> for forty-eight hours, but is destroyed after a longer treatment. The vegetative forms are destroyed in twenty-four hours but the spores survive.

<sup>65</sup> Taylor, E. McK. and Burns, A. C. Egypt. Min. Agr., Tech. Sci. Serv. Bul. 52. 1924.

<sup>66</sup> Gainey, P. L. Mo. Bot. Gard. Ann. Rpt., 23: 147-169. 1912.

<sup>67</sup> Hiltner and Störmer, 1903 (p. 12).

<sup>68</sup> Truffaut and Bezssonoff, 1920-21 (p. 35); Compt. Rend. Acad. Agr. France, 4: 1049-1057, 1030-1038. 1918.

<sup>69</sup> Maassen and Behn, 1924 (p. 731).

A comparison of the influence of antiseptics upon bacteria and protozoa brought out the fact that smaller doses are required to kill the organisms in solution than in soil.<sup>70</sup> The actual concentration of the antiseptic required to destroy amoebae in soil is so large as to become unapplicable for purposes of partial sterilization, which has as its aim the destruction of the protozoa. Even 60 per cent CS<sub>2</sub> did not kill the cysts of amoebae in soil; the same was true of 15 per cent ether, 6 per cent chloroform, 25 per cent CaO, 30 parts per thousand of chlorine water, less than 15 per cent toluol and 5 per cent CaS. Bacterial spores are more resistant to the antiseptic than the cysts of amoebae. Active amoebae have a lower resistance than non-spore-forming bacteria, but the latter are more readily destroyed than cysts of amoebae. The data did not justify any claim for an equilibrium between the numbers of amoebae and bacteria in the soil, the fluctuations of the numbers of amoebae and bacteria being due more to the successive drying and moistening of the test soil than to the action of amoebae on the bacteria.

Various plant pathogenic fungi, particularly organisms like *Fusarium*, *Phytophthora*, *Melanospora*, *Rhizoctonia*, *Neocosmopora vasinfecta*, *Cladosporium scabies*, *Sclerotinia*, *Synchytrium*, spores of rusts and smuts, are readily destroyed by volatile antiseptics, such as toluol, carbon bisulfide, or by heat. A soil containing over 100,000 fungi (spores and pieces of mycelium) per gram becomes practically free from fungi after treatment with steam or volatile antiseptics. Partial sterilization of soil can thus correct the condition of soil sickness caused by the development of certain specific plant pathogens.<sup>71</sup>

Hiltner<sup>72</sup> used a logical process of reasoning for demonstrating the destruction of fungi and actinomyces by antiseptics. When straw is applied to soil, the available nitrogen is stored away by the microorganisms to the detriment of higher plants. When the soil is treated with carbon bisulfide, during or after the addition of straw, the injurious influence is not observed. This is due to the fact that, with straw fertilization, the soil nitrogen is stored away chiefly by fungi and actinomyces. The disinfectant brings about an appreciable reduction in the number of these organisms, thus leaving the soil nitrogen available for higher plants. The favorable influence of CS<sub>2</sub> is due not

<sup>70</sup> Sewertsoff, L. B. Centrbl. Bakt. II, 65: 278-291. 1925; I. Or., 92: 151-158. 1924.

<sup>71</sup> Kaserer, H. Mitt. Wien. Hochschule f. Bodenkultur., 2: 375-410. 1913-14; Russell, E. J. and Petherbridge, F. R. Jour. Board Agr., 19. 1913, No. 10.

<sup>72</sup> Hiltner, 1908 (p. 727).

merely to the destruction of the organisms directly injurious to higher plants but also to the destruction of those which have a passive effect by storing away the soil nitrogen.

Parasitic nematodes, such as *Heterodera radiculicola* and various other worms, are also killed by heating the soil to 60° to 90°C. or by volatile antiseptics like CS<sub>2</sub>.

In view of the fact that actinomyces grow only very slowly and that the injurious factor in the soil is apparently something which is slowly growing, for an infection with 5 per cent of raw soil only begins to show a limiting action upon the fortieth day, Greig-Smith suggested that actinomyces are the limiting factor for bacterial activities. Not all the disinfectants, however, injure the actinomyces alike.

*Summary.*—*Interrelationships of microorganisms in the soil.* If the soil could be imagined in an undisturbed condition, even for a very brief period of time, when the moisture content, aeration and temperature are not changed appreciably, one could speak of a condition of equilibrium. A microbiological equilibrium is distinctly different from that of a chemical reaction; in the latter, equilibrium is reached when the reaction goes both ways at an equal rate. A microbiological equilibrium in the soil may occur when the changes in the numbers and activities of the various groups of organisms are constant, possibly when as many new cells are formed in a given period as are destroyed in that time. An ideal condition of equilibrium may never be reached under field conditions more than for a few brief seconds. Under carefully controlled laboratory conditions, an equilibrium can be readily demonstrated, although it takes a long period of time before it is established.

When a soil is brought into the laboratory and kept at constant optimum moisture and temperature, there is at first a rise in the number of microorganisms, particularly if the soil has been partially or fully air dried before being placed in the incubator. The numbers of microorganisms and the rate of their activities, using the carbon dioxide production of the soil as an index, rise at first, soon drop rapidly and then more slowly, until the rate of change in the numbers and activities becomes constant.

But even under soil conditions, one may speak roughly of a certain equilibrium which becomes established between various groups of microorganisms, in the competition for the available energy, as shown by Hiltner and Störmer. The quantitative and qualitative composition of the soil flora and fauna depend on the amount of energy and nitrogen available as well as the forms in which these are present in the

soil. Any modification in the amount and form of energy and nitrogen brings about a modification not only in the number but also in the kind of microorganisms developing in the soil. Such modifications are brought about either by introducing fresh energy and nitrogen materials, air drying and cultivating of soil, partial sterilization, growth of plants, etc. These modifications can be looked upon as resulting in a shift in the condition of equilibrium.

The differences in the energy and nitrogen metabolism of the various soil microorganisms combined with their relative resistance to the action of disinfectants and their rates of multiplication are the basic factors governing the phenomenon of soil microbiological equilibrium.

The fungi utilize a large amount of the available energy for structural purposes. They produce, therefore, large quantities of carbon dioxide and consume a considerable amount of nitrogen which they transform into microbial protein. They multiply rapidly when large quantities of energy material, like undecomposed organic matter, are added to the soil. They are also less resistant to the action of heat and antiseptics, but multiply very rapidly when reintroduced into partially sterilized soils. In such a case, the fungi, together with the bacteria, contribute a large amount of the carbon dioxide formed, but they use up, for structural purposes, a part of the available nitrogen, which otherwise would remain in the soil as ammonia or nitrates.

The actinomyces develop very slowly, but are more resistant to the action of antiseptics like toluol and air drying of soil. When the soil is kept under uniform conditions and the flora gradually comes to an equilibrium, there is generally an increase in the proportion of actinomyces. When the equilibrium is shifted either by air drying of soil, or by volatile antiseptics, heat, etc., the actual number of actinomyces may diminish only slightly, but after the disinfectant is removed, or when moisture is added to the air dry soil, the actinomyces regain their previous numbers very slowly. In comparison with the rapid increase of the bacteria, they diminish rapidly so that, two years after treatment with carbon bisulfide, they have not fully regained their former numbers. They do not use up much available nitrogen unless an available source of energy is introduced, but a number of them produce substances distinctly toxic to certain bacteria and fungi.

The bacteria are such an heterogeneous group of organisms that their activities cannot be classified together. The spore formers are very resistant to treatments that result in partial sterilization of soil

and may develop abundantly afterwards. The non-spore-forming bacteria are very sensitive to the treatment and are much diminished in numbers as a result of partial sterilization of soil; but soon afterwards they begin to multiply very rapidly and may bring up the numbers to hundreds of millions per gram. They use very little of the energy for structural purposes, and, therefore, consume only very little of the ammonia liberated from the decomposition of the proteins. The ammonia can thus accumulate in the soil, unless the fungi are reintroduced. The temporary suppression of the nitrifying bacteria tends to intensify the accumulation of the ammonia.

The soil protozoa probably also play some part in this group of processes. At least some of the protozoa consume bacteria as food, perhaps even large numbers of them; their selective feeding may affect only certain groups of bacteria. They may thus store away considerable quantities of energy in their bodies. Their direct rôle in definite soil processes has not been established as yet, although some information seems to point to the fact that by removing the old bacterial cells, they may even exert a beneficial effect. The possibility that some protozoa exist as saprophytes in the soil, taking perhaps a part in the decomposition of the soil organic matter, is not excluded.

We may conclude in the words of Miège<sup>73</sup> that disinfection of soil still presents too many obscurities and uncertainties. It is certain that it cannot be presented as a panacea capable of remedying the ills from which agriculture suffers.<sup>74</sup>

<sup>73</sup> Miège, E. *Compt. Rend. Soc. Nat. Agr. France.* March, 1914; *Compt. Rend. Acad. Sci.*, 164: 362-365. 1917.

<sup>74</sup> A review of the literature on soil disinfection is given by Vogt, E. *Centrbl. Bakt. II*, 61: 323-356. 1924.

## CHAPTER XXX

### INFLUENCE OF ENVIRONMENTAL CONDITIONS AND SOIL TREATMENT UPON MICROORGANISMS AND THEIR ACTIVITIES IN THE SOIL

A change in the physical, chemical and physico-chemical condition of the soil brings about a change in the soil flora and fauna. The nature of the change depends upon the treatment. The addition of organic matter stimulates the development of certain specific groups capable of decomposing this particular organic substance. A change in the soil reaction brought about by liming, by addition of sulfur or of ammonium salts favors the development of organisms more adapted to that soil reaction. Excessive rainfall or reduction of pore space by mechanical means modifies the composition of the soil atmosphere and stimulates the development of anaerobic organisms in preference to aerobes. Changes in temperature and aeration also lead to changes in the quantitative and qualitative relationships of the soil population.

Whether we accept the idea of Winogradsky concerning an autochthonous or native soil flora or not, it is certain that a soil, under a given set of conditions, can liberate a definite amount of energy, which is sufficient for the activities of a definite number of microorganisms, the qualitative composition of the population depending upon the soil conditions. Any change in these conditions will modify the population both quantitatively and qualitatively. The changes in the soil population may take place from day to day and even from hour to hour, under field conditions.<sup>1</sup> Samples of soil taken at long intervals of time may not give, therefore, a true picture of the actual soil population and its changes in the soil. It is important to study the soil flora and fauna at frequent intervals and establish the interrelationships between the different members of the population and between the latter and the environmental conditions.

*Influence of organic matter upon the soil population.* The addition of organic matter to soil results in an increase in the numbers of various

<sup>1</sup> Cutler, D. W. and Crump, L. M. Ann. Appl. Biol., 7: 11-24. 1920; Cutler et al., 1922 (p. 29).



groups of soil microorganisms, of which some are directly concerned in the process of decomposition and some utilize the products formed by the first or attack the living or dead cells of those organisms themselves. Differences in the physical and chemical nature of the soil lead to the development of different groups of microorganisms as a result of addition of the same organic substance. The composition of the organic matter is of prime importance in this connection, modifying largely the nature of the organisms which develop in preference to others. The addition of soluble sugars to soil brings about an extensive development of bacteria, primarily nitrogen-fixing forms, such as *Azotobacter*, under aerobic conditions, and *Clostridium* under anaerobic conditions. The addition of substances rich in cellulose and poor in proteins, like cereal straw, stimulates largely the development of fungi and of various aerobic bacteria when the pH of the soil is above 6.0; these decompose the cellulose and synthesize extensive cell substance. This is immediately attacked by a large number of bacteria and actinomyces, leading to the formation of various products, in addition to the synthesized cells of these microorganisms; these, in their turn, along with the residual products of the organic matter and the fungus mycelium, serve as food for protozoa and other invertebrate animals. One group of organisms carries on a certain process and then gives way to another group, which carries on the process further; both may be active at the same time. The activities of these organisms lead to a gradual increase in the carbon content of the residual organic matter, which has become changed into a colloidal mass more or less constant in composition and less readily available as a source of energy. Finally those microorganisms (largely the minute non-spore forming bacteria), which require only a small amount of energy and nutrients, continue to act in the colloidal film surrounding the soil particles, slowly breaking down the residual lignins and the resistant synthesized materials of a protein nature.

Engberding<sup>2</sup> found that the addition of 2 per cent cane sugar to a soil brought about an increase of 1000 to 1500 per cent in the number of bacteria developing upon Heyden agar; 0.5 per cent glucose brought about an increase of only 300 to 400 per cent. This increase was soon followed by a decrease and in some cases there were no more bacteria in the treated soils than in the control soils after two and one-half months.

By direct microscopic examination, it can be demonstrated<sup>3</sup> that the

<sup>2</sup> Engberding, 1909 (p. 14).

<sup>3</sup> Winogradsky, 1924 (p. 10).

addition of glucose or mannitol to soil brings about a rapid development of nitrogen-fixing bacteria; starch stimulates the development of actinomyces, the addition of cellulose brings about an extensive growth of fungi while the addition of dried blood stimulates particularly the spore forming bacteria. These results indicate that the common bacteria are favored primarily by lower carbohydrates and by protein-rich substances, while the fungi and actinomyces are increased to a greater extent by cellulose and other polysaccharides. The greater stimulative effect of sugars upon bacteria than upon fungi and actinomyces is due to several factors:

1. The majority of bacteria prefer glucose to higher carbohydrates and their derivatives, while many fungi readily decompose cellulose, pentosans, etc.; many actinomyces are capable of attacking the lignins in the natural organic materials.

2. Bacteria generally require much less nitrogen for the synthesis of their cells per unit of glucose decomposed than do the fungi, which produce an abundant mycelium requiring considerable nitrogen.

3. Among the bacteria, the nitrogen-fixing forms readily utilize sugars as sources of energy without requiring any combined nitrogen.

The addition of glucose, in the presence of even a small amount of available nitrogen will, therefore, greatly stimulate the development of rapidly growing bacteria and may not affect at all the growth of fungi, which require a large amount of available nitrogen, or of actinomyces, which develop only very slowly.

The stimulative effect of cellulose upon the fungi of the soil is especially marked under aerobic conditions and in the presence of available nitrogen. An extensive bacterial multiplication may take place as a result of addition of cellulose to soil. However, the true cellulose-decomposing bacteria are usually not determined, since they do not develop on the ordinary agar plate. The actual increase in bacterial numbers resulting from addition of cellulose, as shown by the ordinary plate, may be due to the development of bacteria feeding upon synthesized or intermediary products, which result from the activities of fungi and aerobic cellulose decomposing bacteria. Under anaerobic conditions, however, the bacteria are greatly stimulated by the addition of cellulose. Pure cellulose may even depress the development of bacteria which grow on the ordinary plate.<sup>4</sup> Straw, however, stimulates multiplication of bacteria, due to the presence of soluble carbohydrates and proteins.

<sup>4</sup> Hill, H. H. Va. Agr. Exp. Sta. Tech. Bul. 6. 1915.

Plant organic matter, like straw, stubble and green manures, stable manures, as well as organic fertilizers consist of a number of various substances. The addition of this organic matter to soil will stimulate the growth of various groups of organisms.<sup>5</sup> Green manures (rye and vetch) favor the rapid multiplication of bacteria rather than of fungi and actinomycetes. The greater the protein content of the organic materials added to the soil, the greater is the development of bacteria in preference to the fungi, as shown in table 102.<sup>5</sup>

It has been shown elsewhere (p. 613) that the addition of organic matter of a wide carbon-nitrogen ratio to the soil leads to a considerable reduction of the nitrate nitrogen, which results in a harmful effect upon plant growth. However, the following year a beneficial effect may be noted,<sup>6</sup> due to the subsequent decomposition of the synthesized proto-

TABLE 102

*Influence of various substances upon the development of fungi and bacteria (including actinomycetes) in a poor soil*

TREATMENT	INCUBATION	FUNGI	BACTERIA
	days		
Untreated.....		115,700	3,860,000
0.5 per cent glucose.....	2	82,000	22,200,000
Cellulose, 1 per cent.....	17	160,000	3,600,000
Cellulose, 1 per cent + 0.1 per cent NaNO <sub>3</sub> .....	17	4,800,000	4,800,000
Straw, 1 per cent.....	10	600,000	25,200,000
Dried blood, 1 per cent.....	12	1,438,300	473,900,000

plasm. In some cases it has been claimed<sup>7</sup> that the depressing effect of a straw mulch upon nitrate formation is due to the checked evaporation of the soil moisture, which lowers the temperature, prevents the normal air exchange, and creates an unfavorable environment for the formation of nitrates. No attempt, however, has been made to correlate these results with the microbiological activities of the vast soil population, outside of the nitrate-forming bacteria.

*Influence of stable manure.* The introduction of stable manure affects chiefly the following physical, chemical and biological conditions of the soil:

<sup>5</sup> Waksman, S. A. and Starkey, R. L. *Soil Sci.*, 17: 373-378. 1924; Smith, N. R. and Humfeld, H. *Jour. Agr. Res.*, 41: 97-123. 1930.

<sup>6</sup> Bredemann, G. *Landw. Jahrb.*, 43: 669-694. 1913.

<sup>7</sup> Albrecht, W. A. and Uhland, R. E. *Soil Sci.*, 20: 253-268. 1925.

1. Soil temperature. The amount of temperature change depends upon the kind and amount of manure added. The addition of 25 tons of manure per acre may give an average increase of five degrees Centigrade in the temperature of the soil.<sup>8</sup>

2. Soil moisture. A higher moisture holding capacity of the soil results from the addition of manure, because of the accumulation of soil humus. This affects bacterial activities favorably.<sup>9</sup>

3. Soil atmosphere. The rapid decomposition of manure added to the soil results in the formation of large quantities of CO<sub>2</sub>, which tend to improve the physical condition of the soil giving it a crumbly appearance.

4. Reaction and buffer content of soil. The decomposition of available nitrogenous substances (in the liquid part of the urine) leading to the formation of ammonia and nitric acid, on the one hand, and the decomposition of the carbohydrates which may lead to formation of some organic acids, on the other, are important in this connection. The buffering properties of the residual humus are considerable.

5. The introduction of large quantities of readily available energy, as well as of nitrogen and minerals, will in itself greatly stimulate bacterial activities.

6. Finally the introduction of large quantities of living bacteria in the manure may result in a change of the qualitative composition of the soil flora and fauna.

A number of observations have been made concerning the increase in the numbers of bacteria in soil as a result of addition of manure. The numbers depend<sup>10</sup> not only upon the manure added but also upon the cultural methods and crop grown. Fallowing of a soil leads to a decrease in numbers of bacteria as compared with the untreated soil, while manuring and fallowing lead to a decided increase.<sup>11</sup> Chester<sup>12</sup> stated in 1898 that "the greater the organic matter or humus in the soil the greater, *pari passu*, is the number of bacteria." The fertilizing effect of manure, aside from the quantities of actual fertilizer constituents contained within them, was believed to be due merely to the bacterial content of the manure;<sup>13</sup> small applications of manure at frequent intervals rather than large applications made at longer intervals were, therefore, recommended. The bacteria thus introduced

<sup>8</sup> Wagner, F. *Forsch. Agr. Phys.*, 5: 373-402. 1882 (*Centrbl. Agr. Chem.*, 12: 150. 1883); Troop, J. *Ind. Agr. Exp. Sta. 8th Ann. Rpt.*, 18-19. 1895.

<sup>9</sup> Engberding, D. *Centrbl. Bakt. II*, 23: 569-642. 1909; King, W. E. and Doryland, C. J. T. *Kans. Agr. Exp. Sta., Bul. 161*, 211-242. 1909.

<sup>10</sup> Caron, A. *Landw. Vers. Sta.*, 45: 401-418. 1895.

<sup>11</sup> Hiltner and Störmer, 1903 (p. 12); Löhnis, F. *Mitt. Deut. landw. Gesell. H. 364*: 9-17. 1928.

<sup>12</sup> Chester, F. D. *Del. Agr. Exp. Sta. Bul. 40*. 1898; *Bul. 65*. 1904.

<sup>13</sup> Hellström, P. *Exp. Sta. Rec.*, 11: 627. 1900; Stoklasa, J. *Chem. Centrbl. Jahrg.*, 78: (N. F. 11): 1702. 1907; also Stoklasa and Ernest, 1905 (p. 31).

into the soil were considered<sup>14</sup> valuable in bringing about a more rapid decomposition of a green manure crop.

By comparing the influence of manure with inorganic fertilizers, Temple<sup>15</sup> found that the addition of sodium nitrate to soil increased the number of bacteria, as determined by the plate method, from 6,500,000 to 8,480,000; the addition of a complete mineral fertilizer increased these numbers to 11,540,000, while stable manure brought about an increase to 23,310,000, on the average of several determinations. This increase continued over a considerable period. When the manure was previously sterilized, before being added to the soil, the increase in bacteria was even greater than in the case of unsterilized manure. Temple suggested, therefore, that the development of bacteria as a

TABLE 103

*Influence of manure on the development of bacteria in soil, as determined by the plate method*

(5 parts of manure per 100 parts of soil)

INCUBATION	TOTAL NUMBER OF ORGANISMS	INCUBATION	TOTAL NUMBER OF ORGANISMS
<i>days</i>		<i>days</i>	
2	60,000,000	21	50,000,000
3	80,000,000	24	55,000,000
4	125,000,000	29	85,000,000
6	235,000,000	38	45,000,000
9	45,000,000	58	95,000,000
13	43,000,000	94	18,000,000
16	35,000,000	123	20,000,000

result of addition of manure is due to the introduction of the organic matter (available energy) rather than of the bacteria, as suggested by other investigators. A direct relationship between the organic matter added to the soil and the bacterial count was also observed by others.<sup>16</sup> The work of Charpentier and Barthel (p. 388) on the decomposition of pure cellulose in soil tends to confirm the observations that the fertilizing effect of manure is due entirely to its nitrogen and minerals and not to the bacteria introduced. No greater increase in the numbers of bacteria from the addition of stable manure with green manure to

<sup>14</sup> Lipman, J. G. et al. N. J. Agr. Exp. Stat. Bul. 268. 1914.

<sup>15</sup> Temple, J. Ga. Agr. Exp. Sta. Bul. 95. 1911; Centrbl. Bakt. II, 34: 204-223. 1912.

<sup>16</sup> Briscoe, C. F. and Harned, H. H. Miss. Agr. Exp. Sta. Bul., 168. 1915.

the soil was obtained than from the green manure itself.<sup>17</sup> The kind<sup>18</sup> and quantity of manure, as well as the soil conditions,<sup>19</sup> greatly influence the change in microbial activities. Table 103<sup>20</sup> shows that the addition of manure to soil results in an immediate rapid increase in the numbers of microorganisms which reaches a maximum in a few days, and is soon followed by a precipitous decrease, due to the rapid exhaustion of the available organic matter.

*Influence of temperature.* The temperature of the soil varies daily and at different seasons of the year. There is a rise in temperature during the daytime and in the summer, due to heat received from the sun, and a drop during the night and in cold months, due to loss of heat by radiation. The soil does not undergo, however, rapid changes in temperature due to its specific heat. Many of the soil microorganisms can grow at rather wide temperature ranges, and adapt themselves readily to gradual changes in temperature.

The action of temperature upon microorganisms is similar to the action upon a chemical reaction, namely for every increase of 10°C. the reaction increases two to three times, although the range of temperature in the case of organisms is much narrower. Different types of soil organisms grow better at different ranges of temperature. These organisms are usually divided, on this basis, into three groups:

1. *Thermophilic*, or those which require a high temperature for their development, usually 45° to 65°C.
2. *Psychrophilic*, or those which grow best at low temperatures (below 10°C.).
3. *Mesophilic*, or those which grow best at 10° to 45°C.

It is doubtful whether any specific group of "psychrophilic" bacteria exists in the soil, these organisms being more "psychrotolerant." The presence of "thermophilic" groups of organisms is more definite, although most of these are probably also only "thermotolerant" (p. 150, 381). Higher temperatures usually never become limiting factors in the activities of microorganisms in the soil, except in very hot climates, where only the surface layer may be affected. The destructive action

<sup>17</sup> Lemmermann, O. and Einecke, A. Mitt. deut. landw. Gesell., 29: (Stück 52): 702-704. 1914.

<sup>18</sup> Emmerich, R., Graf zu Leiningen, W. and Loew, O. Centrbl. Bakt. II, 29: 668-683. 1911.

<sup>19</sup> Greaves, J. E. and Carter, E. G. Jour. Agr. Res., 6: 889-926. 1916; 9: 293-341. 1917.

<sup>20</sup> Bright and Conn, 1919 (p. 43).

soil of northern Greenland and of the island of Disko near Cape York has been made by Barthel,<sup>29</sup> who found, in addition to various aerobic and anaerobic, spore-forming and non-spore forming bacteria, also actinomyces and fungi. Nitrifying bacteria were present only in three out of fourteen soils of Disko, while *Azotobacter* was absent in all; *Bac. amylobacter* could not be demonstrated, although its presence originally was not excluded; *Azotobacter* and protozoa were found in the Greenland soils.

The great majority of fungi grow at 4.5°C. and even develop slowly at 0°C.<sup>30</sup> The minimum temperatures of germination of fungi vary from 1°C., for *Monilia fructigena* and *Pen. digitatum*, to 6° to 9° for *Cephalothecium roseum*.<sup>31</sup> Spores of *Alternaria*, *Botrytis*, *Pen. expansum* and *Sclerotinia* germinate slowly at zero; the spores of *Ceph. roseum*, *Fusarium radiculicola* and others failed to germinate at zero but germinated slowly at 5°C.<sup>32</sup> Some fungi, like *Pen. expansum*, after starting growth at ordinary temperatures, are able to continue growth at zero. The rate of growth increases with rise in temperature up to a certain limit, being a direct function of  $(t - t^{\circ})$ , where  $t$  is the particular temperature and  $t^{\circ}$  is the minimum temperature.<sup>33</sup>

*Seasonal changes.* At least four different types of variability of the numbers of microorganisms in soil, as determined by a certain procedure, must be recognized. These are as follows: 1. Variability of the methods, including the influence of slight changes in composition of media, methods of making the determination, etc. 2. Natural variability in soil, due to differences in chemical composition between different soil particles and other factors resulting from the non-homogeneousness of the soil. 3. Daily variability. 4. Seasonal variability. The last has received the greatest attention.

The numbers and activities of microorganisms in soil change with the season of the year, due to changes in soil temperature and moisture, introduction of available organic matter, soil aeration, etc. There is usually a rise in autumn and spring, and a drop in winter and in summer. Russell and Appleyard<sup>34</sup> found that there is very little activity in the

<sup>29</sup> Barthel, 1922 (p. 141).

<sup>30</sup> Schneider-Orelli, O. Centrbl. Bakt. II, **32**: 161-169. 1912.

<sup>31</sup> Ames, A. Phytopathol., **11**: 19. 1915.

<sup>32</sup> Brooks, C. and Cooley, J. S. Jour. Agr. Res., **8**: 139-164. 1917.

<sup>33</sup> Brown, W. Ann. Bot., **36**: 257-283. 1922.

<sup>34</sup> Russell, E. J. and Appleyard, A. Jour. Agr. Sci., **8**: 385-417. 1917.

soil during the winter months (November to March). Below 5°C., the changes taking place in soil are very slow; above that temperature, bacterial numbers, CO<sub>2</sub> production, and nitrate accumulation all increase, the curves agreeing closely with the temperature curve. Under favorable temperature conditions, rainfall becomes the limiting factor as well as the oxygen brought down by the rain. In general, a period of spring activity, summer sluggishness, autumn activity, followed by winter inertness were reported. Woitkiewicz<sup>35</sup> also found bacterial numbers to be highest in spring and lowest in winter; the ratios of nitrogen-fixation in solution during the winter, spring, summer, and fall seasons were as 1:2:2 $\frac{2}{3}$ :3, respectively. This may be due to the greater abundance of available energy in the fall of the year. Denitrification was highest in fall and lowest in spring. By the use of the Remy method, it was<sup>36</sup> found that the urea-decomposing, nitrifying, and nitrogen-fixing powers of the soil reached a maximum in the spring, a minimum in summer and a maximum again in autumn. Similar results were obtained in the study of the influence of season of year on ammonia formation from peptone in solution.<sup>37</sup> Maximum nitrifying power in soil was found in spring.<sup>38</sup> According to Lemmermann and Wichers,<sup>39</sup> the season of year, outside of influence of temperature and other physical weathering conditions, has no appreciable influence on nitrification. The seasonal course of ammonia and nitrate formation, with all other factors alike, is influenced primarily by the temperature course;<sup>40</sup> the maximum activity is found in the spring and is due to outer influences, primarily change in temperature, but is independent of inner causes.

Periodic changes have been observed not only in the production and accumulation of nitrates<sup>41</sup> in soil, but also in the evolution of carbon dioxide. In the warmer seasons of the year, the variations in temperature between day and night, as well as weather changes during the day,

<sup>35</sup> Woitkiewicz, A. *Centrbl. Bakt.* II, **42**: 254-261. 1914.

<sup>36</sup> Löhnis, F. and Sabaschnikoff, A. *Centrbl. Bakt.* II, **20**: 322-332. 1908.

<sup>37</sup> Moll, R. *Beiträge zur Biochemie des Bodens.* Leipzig. 1909.

<sup>38</sup> Müntz, A. and Gaudechon, H. *Compt. Rend. Acad. Sci.*, **154**: 163-168. 1912; Lumière, A. *Rev. Gen. Bot.*, **33**: 545-557. 1921; Jensen, C. A. *U. S. Dept. Agr. Bur. Pl. Ind. Bul.* **173**. 1910; Leather, J. W. *Mem. Dept. Agr. India, Chem. Ser.*, **1**: 133-184. 1910; 205-281; **2**: 63-140. 1912.

<sup>39</sup> Lemmermann, O. and Wichers, L. *Centrbl. Bakt.* II, **50**: 33-43. 1920.

<sup>40</sup> Schönbrunn, B. *Centrbl. Bakt.* II, **56**: 545-565. 1922.

<sup>41</sup> Limbach, S. *Centrbl. Bakt.* II, **78**: 354-375. 1929; Buntjakov, S. I. *Zhur. Opit. Agron. Yugo-Vostoka*, **3**: 60-74. 1927.



cause a marked effect upon soil respiration. The same is true of changes in soil moisture, the addition of water bringing about a marked stimulating effect upon  $\text{CO}_2$  evolution which is not lasting, however. While the  $\text{CO}_2$  content of the air in the upper soil layers is parallel to the numbers of microorganisms, this need not hold true for the lower layers, due to diminished gas exchange. Intensive soil cultivation favors considerably  $\text{CO}_2$  evolution. Fallowed soil has the highest respiration, followed by soils growing legumes; soil under cereals possesses lowest respiration. While the  $\text{CO}_2$  content of the air just above the soil is highest, it diminishes in the zone of growing leaves due to the assimilating activities of the latter.<sup>42</sup>

Considerable evidence has thus accumulated that seems to point to the influence of other factors, in addition to temperature changes. Cutler<sup>43</sup> could not trace any connection between the seasonal changes of the protozoan population of the soil and the external conditions including food supply. Some information concerning the daily fluctuation of the numbers of bacteria has already been reported (p. 49). These fluctuations found during the course of a day greatly exceeded the variation between different samples. The numbers were usually high in the morning and low in the middle of the day.<sup>44</sup>

*Influence of moisture.* The influence of moisture upon the activities of microorganisms depends to a large extent upon the nature of the soil and the nature of the organisms. A moisture content sufficient for microbial activities in light sandy soils may be quite insufficient in the case of heavy loam or clay soils. A moisture adequate for certain aerobic bacteria and fungi may prove inadequate for some anaerobic bacteria and certain protozoa. In stating the amount of moisture in a given soil, it is better to express it in per cent of its moisture-holding capacity. The optimum amount of moisture for the activities of most bacteria, fungi and protozoa is between 50 and 67 per cent of this capacity, although growth will also take place at lower and higher moisture concentrations, depending of course upon the nature of the organisms.

Some bacteria, like the nitrifying organisms, are very sensitive to drying, while others, like *Azotobacter*, can resist drying for a long period of time. The addition of moisture to a soil, which contains less water than is the optimum for biological activities, brings about an increase

<sup>42</sup> Johansson, N. Svensk. bot. Tidskr. **23**: 241-260. 1929; Dönhoff, G. Kühn-Archiv., **15**: 457-511. 1927.

<sup>43</sup> Cutler, D. W. Jour. Quekett Micr. Club., **15**: 309-330. 1927.

<sup>44</sup> Thornton, H. G. and Gray, P. H. H. Proc. Roy. Soc., **106B**: 399-417. 1930.

in numbers and activities of microorganisms. Most active nitrification takes place when the soil is allowed to become partially dry between the applications of water;<sup>45</sup> a direct relationship was observed between the speed of nitrification and the moisture content of fallow soil. Every soil has an optimum for nitrification; higher or lower amounts of moisture reduce this optimum. Nitrification is at its highest<sup>46</sup> when the soil contains moisture equivalent to 55.6 per cent of its water-holding capacity. Excessive quantities of water are much more injurious than too small quantities, due to the fact that in the first case soil conditions become anaerobic and denitrification sets in. The water requirements of the microorganisms vary considerably with the soil. Maximum nitrification in a loam soil occurred with 16 per cent water; by reducing the water content to 10 per cent or increasing to 26 per cent, nitrification was greatly retarded.<sup>47</sup>

TABLE 104

*Influence of moisture upon the evolution of CO<sub>2</sub> from different soils*

MOISTURE	CO <sub>2</sub> EVOLUTION FROM LOAM SOIL	MOISTURE	CO <sub>2</sub> EVOLUTION FROM HUMUS SOIL
<i>per cent</i>		<i>per cent</i>	
2.59	100	2.43	100
5.75	397	5.43	207
8.49	554	7.90	329
13.75	767	12.68	254

A definite favorable influence of increased moisture upon bacterial numbers was recorded.<sup>48</sup> Moisture content of the soil may have a greater influence upon bacterial numbers than the temperature. By increasing the moisture content of the soil from 6.5 to 14 per cent, there was an increase in bacterial numbers from 10 to 16.4 millions per gram. By raising the moisture from 6 to 15 per cent, van Suchtelen obtained an increase in CO<sub>2</sub> production from 19 to 208 mgm., which led him to conclude that the latter was a more sensitive method for studying changes in microbiological activities than the determination of numbers, as is shown elsewhere.<sup>49</sup> A definite relation between mois-

<sup>45</sup> Dehérain, P. P. *Ann. Agron.*, 13: 241-261. 1887; also 22: 515-523. 1896.

<sup>46</sup> Fraps, G. S. *Texas Agr. Exp. Sta.*, Bul. 106. 1908.

<sup>47</sup> Coleman, L. C. *Centrbl. Bakt.* II, 20: 401-420, 484-513. 1908.

<sup>48</sup> Fischer, H., Lemmermann, O., Kappen, H. and Blanck, E. *Landw. Jahrb.*, 38: 319-364. 1909.

<sup>49</sup> See also Löhnis, F. *Mitt. landw. Inst. Leipzig*, 7: 1-105. 1905.

ture content of different soils and bacterial activities has been demonstrated.<sup>50</sup> At the lower limits of moisture, less water is required to start nitrification in sand than in clay soils; at the higher limits of moisture, less water is required to stop nitrification in sand than in clay. The optimum for the two soils varies; a rise above the optimum is more injurious than an equal fall below the optimum. Table 104 gives the results obtained<sup>51</sup> on the influence of moisture upon the decomposition of organic matter in the soil, as measured by CO<sub>2</sub> production.

The nature of the soil is also found to be an important factor in modifying the influence of moisture upon microbiological activities as shown in table 104 and in the following summary:<sup>52</sup>

SOIL TYPE	CARBON CONTENT OF SOIL	CO <sub>2</sub> PRODUCED IN 24 HOURS, BY 1 KG.M. OF FRESH SOIL	CO <sub>2</sub> PRODUCED IN 24 HOURS BY 1 KG.M. OF SOIL, STERILIZED AND INOCULATED
	<i>per cent</i>	<i>mgm.</i>	<i>mgm.</i>
Tenacious clay soil.....	1.68	8.2	14.0
Diluvial loam.....	2.12	14.6	27.8
Alluvial soil.....	1.73	36.6	59.8

Winogradsky<sup>53</sup> found that, in the case of a soil with a moisture holding capacity of 48 per cent, 15 per cent moisture favored the development of aerobic bacteria to a depth of 23 cm.; 19 per cent moisture to 18 cm.; 21 per cent moisture caused the anaerobic bacteria to develop a few centimeters below the surface, while 24 per cent moisture and above made the soil decidedly favorable for anaerobic bacteria. Nitrogen fixation is at a maximum<sup>54</sup> near the point of saturation; even higher nitrogen fixation was recorded under anaerobic than under aerobic conditions;<sup>55</sup> in some cases two maxima were observed<sup>56</sup> one under aerobic and the other under anaerobic conditions.

<sup>50</sup> Patterson, J. W. and Scott, P. R. Jour. Dept. Agr. Victoria, 10: 275-282. 1912.

<sup>51</sup> König, J. and Hasenbäumer, J. Landw. Jahrb., 55: 185-252. 1920.

<sup>52</sup> Stoklasa, 1926 (p. XIV).

<sup>53</sup> Winogradsky, 1924 (p. 475).

<sup>54</sup> Traaen, A. E. Centrbl. Bakt. II, 45: 119-135. 1916.

<sup>55</sup> Panganiban, 1925 (p. 748).

<sup>56</sup> Greaves and Carter, 1917 (p. 747); Lipman, C. B. and Sharp, L. T. Bot. Gaz. 59: 402-406. 1915.

Ammonia formation from proteins was found<sup>57</sup> to be at an optimum when the soil contained water equivalent to 60 per cent of its total moisture holding capacity. The formation of ammonia may also be very intensive even in saturated soils.<sup>58</sup> This is due to active decomposition of proteins by anaerobic bacteria. The mechanism of ammonia formation is different of course under aerobic and anaerobic conditions.<sup>59</sup>

In arid regions, the application of irrigation water has<sup>60</sup> a definite beneficial effect upon the number of organisms in fallow soils and upon the ammonifying and nitrifying capacities of both fallow and cropped soils. These activities result in an increase in soluble nitrogen. An excess of water may result in the washing out of the nitrates from the soil. Greaves and Carter, using the Briggs formula for the moisture equivalent and the wilting and hygroscopic coefficients, found that the following equations represent the water requirements for maximum bacterial activities:

$$M_{am} = 0.6c = 0.942e + 12.6 = 1.74w + 12.6 = 2.55h + 12.6$$

$$M_n = 1.55c = 0.8525e + 11.55 = 1.472w + 11.55 = 2.163h + 11.55$$

$$M_{nf} = 0.7c = 1.049e + 14.7 = 1.947w + 1.47 = 2.848h + 14.7$$

$c$  = moisture capacity as defined by Hillgard,  $w$  = wilting coefficient,  $e$  = the moisture equivalent,  $h$  = the hygroscopic coefficient,  $am$  = ammonification,  $n$  = nitrification,  $nf$  = nitrogen fixation.

*Influence of soil cultivation.* Cultivation tends to conserve the soil moisture at a time of the year when it is most needed; it brings about a better aeration of soil, it influences the soil temperature and tends to improve the physical condition of the soil; it brings about a rapid drying of the surface layer of the soil and, when moistened by rainfall, bacterial activities are stimulated. Prolonged drought brings about similar results in a still more pronounced way.<sup>61</sup> Greater numbers of microorganisms were found in cultivated than in uncultivated soils.<sup>62,63</sup> The efficiency of soil for nitrate production is increased by cultivation

<sup>57</sup> Greaves, J. E. and Carter, E. G. *Soil Sci.*, 10: 361-387. 1920; 13: 251-270. 1922.

<sup>58</sup> Lipman, J. G. and Brown, P. E. 29th Ann. Rpt., N. J. Agr. Exp. Sta. 105-115. 1908; Murray, T. J. *Jour. Bact.*, 1: 547-614. 1916.

<sup>59</sup> See also Münter, F. and Robson, W. P. *Centrbl. Bakt.* II, 39: 419-440. 1916; Rahn, O. *Mich. Agr. Exp. Sta. Bul.* 16. 1912; *Centrbl. Bakt.* II, 35: 429-465. 1912; 38: 484. 1913.

<sup>60</sup> Prescott, J. A. *Jour. Agr. Sci.*, 10: 177-181. 1920.

<sup>61</sup> Waite, H. H. and Squires, D. H. *Nebr. Agr. Exp. Sta., 24th Ann. Rpt.*, 160-177. 1911.

<sup>62</sup> Caron, 1895 (p. 745).

<sup>63</sup> Houston, 1898 (p. 14).

and aeration.<sup>64</sup> On comparing the bacteria in soils growing corn and alfalfa, greater numbers of organisms were found<sup>61</sup> to be present in the first three feet of the corn soil than in the same layer of alfalfa soil; this is probably due to better aeration of the first soil brought about by cultivation.

The number of organisms in cultivated soils may be twice as large as in corresponding virgin soils, and higher in wheat land than in alfalfa land.<sup>65</sup> Nitrification and nitrogen-fixation were also twice as active in land under cultivation. The beneficial effect of summer fallowing

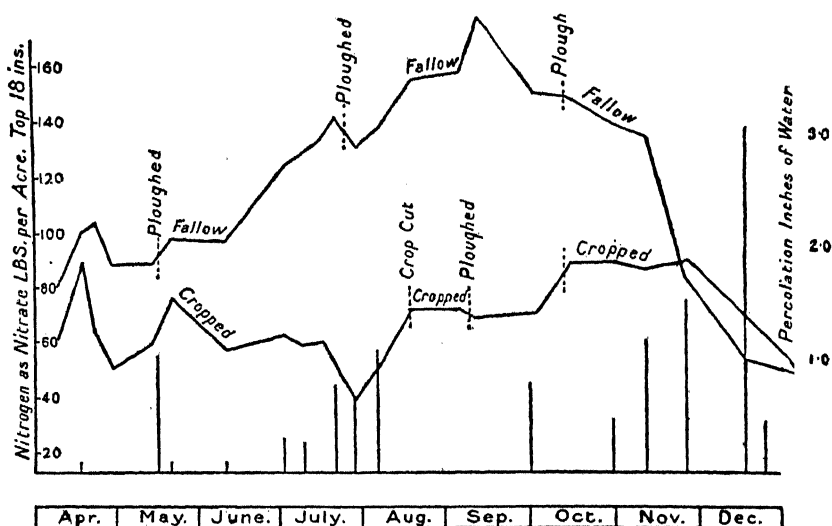


FIG. 77. Influence of cropping and fallowing upon nitrate accumulation in the soil at different seasons of the year (after Russell).

and disking was believed to be due, in part at least, to increased available plant food in cultivated soil, brought about by increased bacterial activities.

Fallowing has a favorable influence on nitrate formation and rapidity of decomposition of organic matter in soil.<sup>66</sup> Caron's results point to a stimulating effect of fallowing upon bacterial numbers, but those of Hiltner and Störmer do not. An increase in the numbers of bacteria

<sup>64</sup> Lyon, T. L. Jour. Amer. Soc. Agron., 14: 97-109. 1922.

<sup>65</sup> Greaves, J. E. Centrbl. Bakt. II, 41: 444-459. 1914.

<sup>66</sup> Ehrenberg, P. Fühlings landw. Ztg., 58: 241-246. 1909.

followed later by a decrease, as a result of fallowing, has also been reported.<sup>67</sup> Several times as much carbon dioxide was found<sup>68</sup> in fallowed land than in corresponding land left under grass; however, in soils poor in inorganic matter carbon dioxide may soon become lower in the fallowed soil. Nitrate formation<sup>69</sup> and nitrogen-fixation are favorably influenced by cultivation and fallowing. It has been observed at an early date<sup>70</sup> that fallowing makes the soil poorer in carbon but relatively richer in nitrogen, due to the greater decomposition of the organic matter.

The nature of the crop rotation also influences the activities of microorganisms in soil; a soil under a four year rotation, while the fourth crop was still on the land or before the cycle was completed for the first time, gave an increased number of organisms over that of the soil under continuous cropping. Soil under continuous corn and wheat contained relatively low numbers of bacteria, in comparison with the timothy and rotation plots.<sup>71</sup>

When a soil is water-logged<sup>72</sup> it becomes deficient in nitrogen. Such soil presents an unfavorable medium for the growth of most fungi; it is favorable to the development of anaerobic, nitrate and sulfate reducing bacteria. Various aerobic bacteria and protozoa are still capable of living under reduced oxygen pressure. A small concentration of CO<sub>2</sub> in soil has even a favorable influence upon the activity of microorganisms.<sup>73</sup>

*Influence of salt concentration upon the activities of microorganisms in the soil.* The addition of mineral nutritive elements to the soil influences the activities of microorganisms in various ways. (1) It stimulates the growth of higher plants, thus leading to an increase in crop residues, greater supply of available energy and, therefore, an increase in microbial activities. (2) In the presence of an excess of available energy, the mineral elements are often limiting factors in the activities of microorganisms; this is true especially of nitrogen, phosphorus,

<sup>67</sup> Krüger, W. and Heinze, B. Landw. Jahrb., 36: 383-423. 1907; A detailed study of the influence of fallowing upon plant growth and bacterial activities has been made by Makkus, W. Landw. Jahrb., 47: 673-718. 1914; Pfeiffer, Th. Landw. Vers. Sta., 98: 187-222. 1921.

<sup>68</sup> Wollny, 1897 (p. 417).

<sup>69</sup> Dehérain, 1896 (p. 753).

<sup>70</sup> Boussingault, J. B. Compt. Rend. Acad. Sci., 48: 303-318. 1859.

<sup>71</sup> Brown, P. E. Iowa Agr. Exp. Sta., Res. Bul. 6. 1912; Gainey, P. L. and Gibbs, W. M. Jour. Agr. Res., 6: 953-975. 1916.

<sup>72</sup> Subrahmanyam, V. Jour. Agr. Sci., 17: 429. 1927.

<sup>73</sup> Rippel, A. and Heilmann, F. Arch. Mikrob., 1: 119-136. 1930.

potassium, calcium and magnesium. (3) It tends to produce a more favorable balance in the concentration of the soil solution and colloidal condition of the soil, for these activities.<sup>74</sup> Too great a concentration of salts, however, as in the case of saline and alkali soils, tends to injure

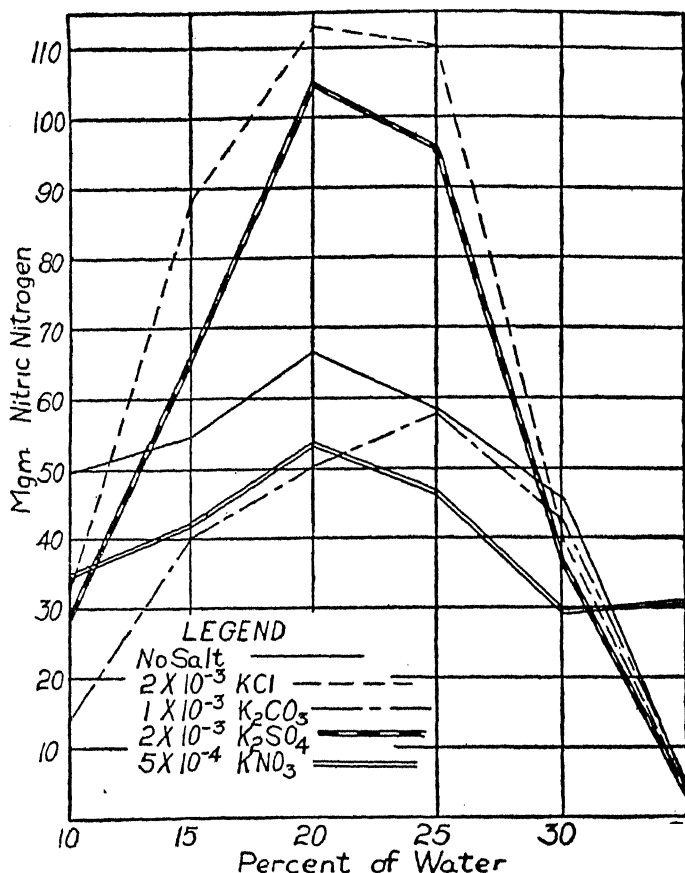


FIG. 78. Influence of moisture and potassium salts upon nitrate accumulation in the soil (from Greaves and Carter).

these activities, although most soil microorganisms are capable of withstanding fairly high salt concentrations (fig. 78).

Phosphoric acid has a favorable influence upon the development of

<sup>74</sup> See also Demeter, K. J. Fortschr. Landw., 2: 69-71. 1927.

*Azotobacter*.<sup>75</sup> The growth of this organism is in a certain way proportional to the easily soluble phosphates in the soil.<sup>76</sup> The amount of nitrogen fixed in a phosphorus-free mannitol solution, to which a definite amount of soil is added, is frequently used (p. 709) as an index of the available phosphorus in the soil. The addition of soluble phosphates to soil may bring about a large increase in the number of soil bacteria and in the decomposition of organic matter, as measured by the formation of ammonia and carbon dioxide; sulfates exert only a limited effect.<sup>77</sup> The possibility that the increased crop production resulting from the application of phosphates to the soil is due, in part at least, to the stimulation of bacterial activities was, therefore, suggested. The effect of addition of  $\text{NaNO}_3$ ,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{K}_2\text{SO}_4$ ,  $\text{MgSO}_4$  to soil upon the numbers of bacteria,<sup>78</sup> is limited. Calcium cyanamide is claimed to have a specific stimulating effect upon soil bacteria, favored by a neutral or alkaline soil condition.<sup>79</sup>

The continuous applications of large quantities of artificial fertilizers may injure certain groups of bacteria, particularly the nitrate forming organisms, which may be overcome by the nitrite formers.<sup>80</sup> Many bacteria and fungi can readily withstand high concentrations of salts, varying from 5 to 15 per cent of sodium chloride, depending on the nature of the salt and the type of the organism. The influence of alkali salts upon bacterial activities in soil received special consideration.<sup>81</sup> Chlorides are usually most toxic, followed by nitrates, sulfates and carbonates. There is a definite antagonism between various ions;<sup>82</sup>

<sup>75</sup> Heinze, B. *Landw. Jahrb.*, **35**: 889-910. 1906; Wilfarth, H. and Wimmer, G. *Landw. Vers. Sta.*, **67**: 27-50. 1907.

<sup>76</sup> Lipman, J. G. *N. J. Agr. Exp. Sta. Ann. Rpt.*, **19**: 177-187. 1906.

<sup>77</sup> Fred, E. B. and Hart, E. B. *Wis. Agr. Exp. Sta. Res. Bul.*, **35**. 1915; *Centrbl. Bakt. II*, **45**: 379. 1916.

<sup>78</sup> Engberding, 1909 (p. 14).

<sup>79</sup> Kuhn, J. and Drecksel, O. *Ztschr. Pflanzen. Düng. Bodenk.*, **7B**: 105-118. 1928; Wolff, A. and Wolff, G. *Centrbl. Bakt. II*, **81**: 221-230. 1930.

<sup>80</sup> Aberson, Y. H. *Meddel. Rijks. Hoog. Hand. Boschborowsch.*, **11**: 1-93. 1916 (*Physiol. Abstr.*, **1**: 509. 1917); see also Wolff, A. *Centrbl. Bakt. II*, **70**: 41-45. 1927.

<sup>81</sup> Lipman, C. B. *Centrbl. Bakt. II*, **32**: 53-64. 1912; **33**: 305-313, 647-655. 1912. Greaves, J. E. *Soil Sci.*, **2**: 443-480. 1916; *Jour. Agr. Res.*, **16**: 107-135. 1919; Kelley, W. P. *Jour. Agr. Res.*, **7**: 417-437. 1916; Prikhodko, M. M. and Belikova, M. M. *Pedology*, 1929: 145-167.

<sup>82</sup> Lipman, C. B. *Bot. Gaz.*, **48**: 106. 1909; *Centrbl. Bakt. II*, **41**: 430-444. 1914; Greaves, J. E. *Soil Sci.*, **10**: 77-102. 1920; Szücs, J. *Jahrb. wiss. Bot.*, **52**: 85-142. 1912.



the nature of the organism and the process studied are of great importance in measuring the antagonistic effect.

In suitable concentrations, salts of arsenic, copper, lead, zinc, and iron, stimulate the activities of the nitrifying and other bacteria.<sup>83</sup> Here as well, the action depends on the nature of the salt, type of soil and nature of organism. Other investigators<sup>84</sup> recorded, however, that  $\text{As}_2\text{O}_3$  in itself does not stimulate bacterial growth. The effect of boron upon legume bacteria consists largely in stimulating the plant to supply the required energy for the bacteria.<sup>85</sup>

The organisms that decompose proteins with the formation of ammonia are apparently more resistant to the action of salts than are the higher plants. Nitrates are toxic to bacteria in definite concentrations, the limits depending on the organism and the medium;<sup>86</sup> this is true of various autotrophic bacteria, independent of the ability of the organisms to assimilate nitrate nitrogen. The activities of the majority of heterotrophic organisms are favorably influenced by small amounts of nitrate, due to the fact that it serves as a good source of nitrogen, in the presence of available energy.

*Influence of calcium oxide and of carbonates of calcium and magnesium.* The influence of calcium oxide and calcium carbonate on bacterial numbers and activities depends largely on the change produced in the soil reaction. In the case of acid soils, there is a decided stimulating effect as a result of application of lime; this effect is also due to a change in the physical condition of the soil which becomes a more favorable medium for the growth of bacteria.

Ramann and associates<sup>87</sup> have shown in 1899 that bacteria predominate in alkaline and neutral soils, while in acid soils, fungi are more predominant and may even exceed the bacteria, especially in acid peat soils. Treatment of soil with fertilizers, such as ammonium sulfate, acid phosphate, sulfur, which tend to leave the soil reaction acid, tends to bring about an increase in the number of fungi and a decrease in

<sup>83</sup> Greaves, J. E. and Carter, E. G. Bot. Gaz., 77: 63-72. 1924; Centrbl. Bakt. II, 39: 542-560. 1913; 42: 244-254. 1914; Jour. Agr. Res., 6: 389-416. 1913; Lipman, C. B. and Burgess, P. S. Univ. Cal. Publ. Agr. Sci., 1: 127-139. 1914.

<sup>84</sup> Cobet, R. and van der Reis, V. Biochem. Ztschr., 129: 73-88. 1922.

<sup>85</sup> Brenchley, W. E. and Thornton, H. G. Proc. Roy. Soc. 98B: 373-399. 1925. Effect of cyanides upon bacteria is discussed by Burnet, F. M. Jour. pathol. Bact., 30: 21-38. 1927.

<sup>86</sup> Böttger, H. Centrbl. Bakt. II, 54: 220-261. 1921.

<sup>87</sup> Ramann, E., Remele, E. Schellhorn and Krause, M. Ztschr. Forst. u. Jagdwes., 31: 577-606. 1899.

the number of bacteria, especially when there is not sufficient lime to neutralize the acid. A definite increase in the number of bacteria, as a result of addition of lime to acid soils, has been observed repeatedly.<sup>88</sup> It was concluded that small applications of  $\text{CaCO}_3$  are more effective, as a rule, than large applications in stimulating bacterial activities; the stimulating effect was obtained, however, only after the neutral point has been reached, as shown by the Veitch method. This is not necessarily true for all soils, since in the case of some acid soils a decided stimulating effect of lime upon the numbers of bacteria is found even if the pH is not above 6.3.

The addition of  $\text{CaO}$  and  $\text{MgO}$  to soil results in a reduction in the carbon dioxide formation until the oxides are all carbonated; this is followed by an increase, as in the case of the carbonates.  $\text{CaSO}_4$  seems to have no appreciable influence on bacterial activities in soil.<sup>89</sup> When 0.5 gram  $\text{CaO}$  and 0.75 gram hay were added to 1 kgm. of soil, the unlimed soil produced, in 18 days, 380 mgm.  $\text{CO}_2$  more than the limed soil (theoretical amount necessary to carbonate the  $\text{CaO}$  is 390 mgm.). After that period, the  $\text{CaO}$  treated soil began to give increased amounts of  $\text{CO}_2$ .<sup>90</sup> The addition of lime increases the decomposition of the organic matter added to the soil, whether this is determined by the evolution of carbon dioxide or formation of nitrates. The increase in the decomposition of the soil organic matter as a result of addition of lime depends on the nature of the soil and the change in reaction brought about, rather than upon the nature of the organic matter added.<sup>91</sup> In this connection, the following results may be cited:<sup>92</sup>

*Influence of  $\text{CaCO}_3$  on evolution of  $\text{CO}_2$  from soil*

$\text{CaCO}_3$ ADDED	$\text{CO}_2$ EVOLVED PER DAY
<i>per cent</i>	<i>mgm.</i>
0	181.3
0.04	223.6
0.10	308.4
0.20	416.4
0.40	455.4

<sup>88</sup> Fischer, H. Landw. Vers. Sta., 70: 335. 1909; Bear, F. E. Soil Sci., 4: 433-462. 1917; Hutchinson, H. B. and McLennan, K. Jour. Agr. Sci., 7: 75-105. 1915.

<sup>89</sup> Cubbon, M. H. Cornell Univ. Agr. Exp. Sta. Mem., 97. 1926.

<sup>90</sup> Lemmermann, O., Aso, K., Fischer, H. and Fresinius, L. Landw. Jahrb., 41: 217-256. 1911.

<sup>91</sup> Miyake and Nakamura, 1923 (p. 612); White, J. W. and Holben, F. J. Soil Sci., 20: 313-327. 1925.

<sup>92</sup> König and Hasenbäumer, 1920 (p. 754).

The addition of an excess of calcium carbonate may be detrimental to certain bacterial activities, as shown by Wollny for the decomposition of organic matter and by Lipman and associates<sup>93</sup> for ammonia formation. In many cases, however, beneficial results may be obtained.<sup>94</sup> An excess of calcium oxide may exert a sterilizing effect upon the soil (p. 720). Magnesium carbonate becomes toxic in large amounts, although in small quantities it may be more effective than calcium carbonate.<sup>95</sup>

By modifying the reaction of the soil, one can exert a selective influence upon the soil population.<sup>96</sup> An excess alkalinity or an excess acidity will favor or depress the action of various beneficial and injurious soil microorganisms.

*Influence of radiation upon the activities of microorganisms.* In addition to the effects of temperature, moisture, aeration and reaction, as well as the presence of various nutrients, both organic and inorganic, microorganisms are affected by a number of other factors. Here belong the stimulating or injurious action of various chemical substances, frequently present in mere traces, and the action of different rays. These differ markedly in their action upon bacteria.<sup>97</sup> This would seem to be of special interest since Stoklasa has shown that the soil atmosphere possesses radioactive properties:  $\alpha$ -rays, in the form of air-containing emanation, stimulate the decomposition of sugar and of nitrogen assimilation by *Azotobacter*, although amounts above a certain limit retard the assimilation process;  $\beta$  and  $\gamma$  rays retard nitrogen assimilation in liquid media, but stimulate the decomposition of proteins by enzymes.

<sup>93</sup> Lipman, J. G., Brown, P. E. and Owen, I. L. *Centrbl. Bakt.* II, **30**: 156-181. 1911.

<sup>94</sup> Peck, S. S. *Hawaiian Sugar Planter's Chem. Bul.*, **34**. 1911; Greaves, J. E. *Soil Sci.*, **2**: 443-480. 1916; Fulmer, H. L. *Jour. Agr. Res.*, **12**: 463-504. 1918. Further information on the influence of calcium upon the activities of microorganisms is given by Miller. *Ztschr. Gärungsphysiol.*, **4**: 194. 1914; Plummer, J. K. *Jour. Amer. Soc. Agron.*, **13**: 162-171. 1921.

<sup>95</sup> Lipman, J. G. and Brown, P. E. *N. J. Agr. Exp. Sta.* 28th Ann. Rpt., 141-204. 1907.

<sup>96</sup> Remy, Th. *Deut. landw. Presse* **53**: 514. 1926.

<sup>97</sup> Stoklasa, J. and Kricka, J. *Centrbl. Bakt.* II, **74**: 161-183. 1928.

## CHAPTER XXXI

### HIGHER PLANTS AND SOIL MICROORGANISMS—MUTUAL INTERRELATIONSHIPS

The relation between higher growing plants and soil microorganisms embraces several distinct phenomena: 1. plants and microorganisms influence one another by the production of certain growth stimulating substances; 2. as a result of the activities of one group, substances are formed which become direct nutrients for the other group; 3. they may actually live together in symbiotic relationships; 4. they may compete with one another for certain nutrients present in the soil; 5. one may become injurious to the other, by direct attack or by the action of certain toxic substances produced by either organism.

*Influence of soil microorganisms upon plant growth.* Microorganisms take part in various soil processes which directly affect the growth of higher plants. They decompose the soil organic matter and liberate the nitrogen and minerals necessary for the growth of higher plants; they also produce considerable quantities of  $\text{CO}_2$ , which is essential for the growth of these plants. They oxidize and otherwise transform the various minerals introduced into the soil (ammonium salts, sulfur), or formed from the decomposition of the organic matter (as  $\text{NH}_3$ ,  $\text{H}_2\text{S}$ ), into forms readily available to plants. They synthesize organic matter from inorganic compounds and thus compete with higher plants for the available nitrogen and minerals;<sup>1</sup> this process may become useful in the absence of a growing crop, since the soluble materials are prevented from being leached out. They reduce, under proper conditions, various oxidized substances like sulfates and nitrates to substances which may be directly toxic to higher plants. They enter into various associations with plants, which are quite important in the growth of the latter. They may actually attack the higher plant and cause their destruction.

The growth of the extensive group of leguminous plants is directly affected by the symbiotic nodule-forming bacteria, so much so that these plants become almost independent of the soil nitrogen and, therefore, of all processes affecting the available nitrogen in the soil. The growth

<sup>1</sup> Chouchack, D. Compt. Rend. Acad. Sci., 185: 82-85. 1927.

of a large number of trees and other plants depends to a large extent upon the fungi forming mycorrhiza on their roots. Whatever the nature of the phenomenon, whether it is a case of symbiosis or of mutual parasitism, there is no doubt that the fungi favor in some way the growth of the plants, both in the case of ectotrophic and endotrophic forms. The hypotheses concerning the rôle of fungi in tuberization in plants as well as in protein formation in certain seeds belong to the same group of relationships.

In addition to legume bacteria, various other bacteria are capable of penetrating the roots of plants and developing there; it is claimed that, here as well, we are dealing with a symbiotic relationship ("bacteriorrhiza") similar to that of mycorrhiza.<sup>2</sup> Certain soil organisms are capable of bringing about a decided stimulation of the development of plant roots; the rôle of this phenomenon in plant growth still remains to be determined.<sup>3</sup>

Microorganisms affect the growth of higher plants not only directly, but also indirectly. The formation of carbon dioxide and various organic acids brings about a greater solubility of the soil minerals, particularly the carbonates and phosphates, as well as to some extent the zeolitic materials. To this we must add, of course, the action of inorganic acids, namely nitrous, nitric and sulfuric, which result directly from the activities of microorganisms. In view of the fact that microorganisms greatly influence the concentration of gases in the soil atmosphere, especially of oxygen and carbon dioxide, root development of higher plants may be appreciably affected; oxygen pressure is known to be of great importance in this connection.<sup>3a</sup>

The favorable influence of legumes upon non-leguminous plants may also be noted here.<sup>4</sup> This led Lipman to conclude that soluble material is excreted by the roots of legumes, either as a result of root decomposition or simple excretion.

<sup>2</sup> Hiltner, L. Arb. deut. landw. Gesell., **98**: 59-78. 1904; Centrbl. Bakt. II, **14**: 46-48. 1904; Perotti, R. Proc. Int. Soc. Soil Sci. **2**: 146-161. 1926.

<sup>3</sup> Riker, A. J., Banfield, W. M., Wright, W. H. and Keitt, K. G. Science, **68**: 357-359. 1928.

<sup>3a</sup> Cannon, W. A. Physiological features of roots. Carnegie Inst. Washington, Publ., **368**. 1925.

<sup>4</sup> See also Koch, A. Chem. Ztg., **36**: 726. 1911; Koch, A. Centrbl. Bakt. II, **41**: 545-572. 1913; Gibbs, W. M. and Werkman, C. H. Soil Sci., **13**: 303-322. 1922; Bondorff, K. A. Den. Kgl. Verter. Z. Lanboh. Aarskr. 1918, 339-362 (Physiol. Abstr., **6**: 137); Lipman, J. G. Jour. Agr. Sci., **3**: 297-300. 1909; N. J. Agr. Exp. Sta. Bul., **253**. 1912; Lyon, T. L. and Bizzell, J. A. Jour. Ind. Eng. Chem. **2**: 313-315. 1910; N. Y. (Cornell) Univ. Agr. Exp. Sta. Bul., **294**. 1911.

When seeds are planted immediately after turning under a green manure crop, the seedlings may be injured. This may be due partly to the action of microorganisms: as a result of decomposition of the green manure, numerous fungi develop, some of which are destructive to seedlings, especially in the case of oil seeds; the rapid evolution of  $\text{CO}_2$  and utilization of oxygen produce conditions unfavorable to oxidation, which is essential for the seeds in the process of germination. However, when seeds are planted two weeks after the addition of the green manure, there is no serious injury to germination.<sup>5</sup>

An attempt was made to explain unproductiveness of soils not by a lack of proper nutrients but by the presence of substances actually injurious to plant growth;<sup>6</sup> these substances were presumably formed in the soil, partly at least, as a result of activities of microorganisms. The production by microorganisms of plant stimulating substances, or "auximones" (p. 370) is still a matter of conjecture. However, we must recognize the fact that, under certain conditions at least, the presence of decomposed organic residues and of microbial cell substance is of considerable importance in favoring growth of plants and microorganisms. Whether this is due to a "buffering" or "poising" effect upon the oxidation-reduction potential of the medium, or to the production of a "bios" or stimulating substance, whatever its nature may be, remains to be determined.

*Influence of nitrogenous decomposition products on the growth of plants.* As a result of the activities of microorganisms, proteins give rise to a large number of substances. The most important of these is ammonia. This is either assimilated without change by plants or microorganisms or is first converted into nitrates by the nitrifying bacteria. In addition to ammonia, other nitrogenous compounds formed from the decomposition of proteins by microorganisms are beneficial to the growth of higher plants.<sup>7</sup> It has even been observed that substances such as nucleic acid, hypoxanthine, guanine, histidine, arginine and creatinine, are absorbed directly by the plant, without first being transformed into ammonia and nitrates. Collectively these compounds were found to be more beneficial than when used singly.<sup>8</sup> It is possible that these sub-

<sup>5</sup> Fred, E. B. Jour. Agr. Res., 5: 1161-1176. 1916.

<sup>6</sup> Schreiner, O. and Reed, H. S. U. S. Dept. Agr. Bur. of Soils, Bul. 40. 1907.

<sup>7</sup> Schreiner, O., Reed, H. S. and Skinner, J. J. Bur. Soils, U. S. Dept. Agr. Bul., 47. 1909; Schreiner, O. and Lathrop, E. C. Jour. Amer. Chem. Soc., 34: 1242-1259; Skinner, J. J. Bur. Soils, U. S. Dept. of Agr. Bul., 83. 1911; Hutchinson, H. B. and Miller, N. H. J. Centrbl. Bakt. II, 30: 513-547. 1911.

<sup>8</sup> Schreiner, O. and Skinner, J. J. Bur. Soils, U. S. Dept. Agr. Bul., 87. 1912.

stances, especially the nucleic acids, are not used as nutrients directly, but play a rôle in the growth of plants and microorganisms similar to that played by vitamins in the growth of higher plants.<sup>8a</sup>

Some of the decomposition products may have a harmful effect upon plant growth,<sup>9</sup> as in the case of the various nitrogenous and non-nitrogenous substances that can be isolated from the soil, including pyridine and its derivatives, dihydroxystearic acid, the so-called "gliedine" that is formed in peat soils,<sup>9a</sup> etc.

*Influence of growing plants upon soil microorganisms.* The growing plants exert various influences upon the activities of the microorganisms in the soil:

1. They secrete soluble substances which offer a favorable medium for the growth of microorganisms.<sup>10</sup> Under sterile conditions, plants secrete formic, oxalic and malic acids, as well as reducing and non-reducing sugars.<sup>11</sup> Plant roots also secrete phosphatides and stimulate the development of various soil fungi.<sup>12</sup>

2. They supply energy and nitrogen sources for the microorganisms through their residues, in the form of dead roots and root hairs, root cap cells, epidermal cells, etc.<sup>13</sup>

3. They remove the various soluble materials from the soil through their roots, especially the nitrates. This changes the composition of the soil solution and modifies the activities of microorganisms.<sup>14</sup>

4. They excrete carbon dioxide into the soil, which, in addition to that produced by the microorganisms, tends to change the reaction

<sup>8a</sup>Rowlands, M. J. and Wilkinson, B. *Biochem. Jour.* **24**: 199. 1930.

<sup>9</sup>Schreiner, O. and Shorey, E. C. *Bur. Soils, U. S. Dept. of Agr. Bul.* **53**. 1909; **74**. 1910.

<sup>9a</sup>Smith, W. S. *Diss. Wageningen.* 1927.

<sup>10</sup>Wilson, J. K. *N. Y. (Cornell) Univ. Agr. Exp. Sta. Mem.*, **65**. 1923; Lyon, T. L. and Wilson, J. K. *Ibid.*, **40**. 1921.

<sup>11</sup>Czapek, F. *Jahrb. wiss. Bot.*, **29**: 321-390. 1896; Mazé, P. *Ann. Inst. Past.*, **25**: 705-738. 1911; Schulov, I. C. *Investigations on the physiology of nutrition of higher plants.* Moskau. 1913; Sabinin, D. A. and Minina. *Bull. Inst. Biol. Res. Univ. Perm.* **5**: 233-258. 1927; **6**: 165-192. 1928; Demidenko, T. *Nauch. Agron. Zhur.*, **5**: 528-540. 1928.

<sup>12</sup>Cranner, B. H. *Meld. Norges Landbrucks*, **1-2**: 1-160. 1922; Melin, E. *Svensk. Bot. Tidskr.*, **18**: 460-464. 1924.

<sup>13</sup>Weaver, J. E. *Root development of filed crops.* McGraw Hill Book Co. 1926.

<sup>14</sup>Wilfarth, H., Römer, H. and Wimmer, G. *Landw. Vers. Sta.*, **63**: 1-70. 1905.

of the soil,<sup>15</sup> increases the solubility of certain inorganic soil constituents and changes the composition of the soil atmosphere.

5. They remove the moisture from the soil, exerting an injurious influence upon the growth of microorganisms. The importance of this influence was, however, minimized by Lyon and associates.<sup>16</sup>

6. Plant roots modify the structure of the soil and produce a medium more favorable for the development of microorganisms. The removal of nitrates from the soil, leaving the bases behind in the form of carbonates, would come under the third group of phenomena; this may affect the activities of microorganisms favorably.<sup>17</sup>

The influence of growing plants upon bacterial activities may be expressed in terms of (1) numbers of microorganisms, (2) nitrate accumulation or nitrifying capacity of the soil, (3) oxidizing power of the soil as expressed either in terms of oxygen absorption or carbon dioxide production, (4) other biological activities. It is not a simple matter to determine the influence of the growing plant upon the numbers of microorganisms, due to many variables involved in a study of this kind and the fact that the results are very difficult of duplication; it is also difficult to differentiate between the direct influence of the growing plant and the influence of the plant products.

Schultz-Lupitz<sup>18</sup> was the first to call attention to the fact that some plants leave the soil in a more fertile condition for the growth of other crops and other plants leave it in a less fertile condition. Without knowing as yet the rôle of microorganisms in the fixation of nitrogen by leguminous plants, he divided the plants in general into nitrogen-consumers and nitrogen-enrichers. Caron<sup>19</sup> argued that if we assume that the existence of bacteria in soil has a favorable influence upon the growth of agricultural plants, we may also expect that the reverse will hold true, namely that plants will exert a favorable influence upon the development of bacteria; he believed that such improvement can be accomplished by means of soil inoculation with known cultures of bacteria, assuming thereby that these are beneficial for certain plants.

<sup>15</sup> Hall, A. D. and Miller, N. H. J. *Proc. Roy. Soc.*, **77B**: 1-32. 1905; Mayers, H. *Wiss. Arch. Landw. A*, **2**: 472-544. 1929.

<sup>16</sup> Dehérain, P. P. *Traité de Chimie Agricole*, 586-587. 1902; Lyon, T. L., Heinicke, A. J. and Wilson, B. D. *N. Y. (Cornell) Univ. Agr. Exp. Sta. Mem.*, **63**. 1923; *Mem.*, **91**. 1925.

<sup>17</sup> Berkmann, M. *Intern. Mitt. Bodenk.*, **3**: 1-49. 1913; Hall and Miller. 1905.

<sup>18</sup> Schultz-Lupitz. *Landw. Jahrb.*, **10**: 777-848. 1881.

<sup>19</sup> Caron, A. *Landw. Vers. Sta.*, **45**: 401-418. 1895.



Hiltner applied the term *rhizosphere* to designate that portion of the soil where the microorganisms are subjected to the action of plant roots; he believed that root excretions favor bacterial growth. Plants were believed to favor the development of those bacteria which are necessary for their growth and nutrition. Due to differences in root excretions and changing conditions of nutrition, each plant, under different conditions, was considered as able to establish a distinct relationship with bacteria. The various claims for the improved growth of non-leguminous plants, such as tobacco, corn, mustard, due to inoculation with *Azotobacter* or other organisms isolated from the rhizosphere of these plants were based upon this assumed relationship between the plants and the bacteria.<sup>20</sup>

According to Löhnis,<sup>21</sup> bacterial numbers increase as a result of plant growth, especially in soils growing legumes; the results are even more marked during the second year than during the first. A larger number of organisms was found<sup>22</sup> under clover than under grain crops, one gram of clover soil containing seven to eight millions of microorganisms, barley soil five to six millions, and soil growing sugar beets one to two millions. Higher numbers of bacteria were found in soil under cowpeas than in fallow land.<sup>23</sup> Wilson<sup>24</sup> placed 300-gram portions of soil in large test tubes; these were plugged with cotton and sterilized. Some were inoculated with sterile corn and some not. A few of the tubes with and without corn were inoculated with a nitrate reducing organism, a few with *Bact. radicicola*, and a few left uninoculated. After 25 to 75 days incubation, the numbers of bacteria were determined in the various soils. About three times as many bacteria were found in the planted as in the unplanted soil, irrespective of the organism and of the nitrate added to the soil. Soils adjacent to the roots of various plants had, in 27 out of 32 soils, a higher bacterial content than the soil at some distance away from the roots.<sup>25</sup>

<sup>20</sup> Hiltner, L. Centrbl. Bakt. II, 58: 351. 1921; Joshi, N. V. Mem. Dept. Agr. India, Bact. Ser., 1: 247-276. 1925; Kas, V. Bull. Czechoslov. Acad. Agr. 5: 861-865. 1929; Kostytschew, S. Proc. 2d Intern. Congr. Soil Sci., 3rd Comm. 1930; Truffaut, G. and Bezssonoff, N. Compt. Rend. Acad. Biol., 91: 1077-1078. 1924.

<sup>21</sup> Löhnis, F. Soil Sci., 22: 365-389. 1926.

<sup>22</sup> Stoklasa and Ernest, 1905 (p. 31); Greuzburg, U. Landw. Jahrb., 68: 75-116. 1928.

<sup>23</sup> Lechair, C. A. Jour. Agr. Res., 5: 439-448. 1916.

<sup>24</sup> Wilson, J. K. and Lyon, T. L. N. Y. (Cornell) Univ. Agr. Exp. Sta. Mem., 103. 1926.

<sup>25</sup> Hoffman, C. Kansas Univ. Science Bul., 9: 81-99. 1914; see also Vander-velde, J. J. and Verbelen, A. Compt. Rend. Acad. Sci., 190: 977-979. 1930.

Among the bacteria which are particularly influenced by the growing plants, the *Bact. radiobacter* group occupies a prominent place. To determine the occurrence and abundance of these organisms, the soil is diluted to 1:10,000 or more. To several of the higher dilutions, 0.5 cc. of a 0.1 per cent crystal violet solution is added and 1 cc. portions plated out, within 2-3 minutes, on a glycerol-nitrate agar (1000 cc. soil extract (1 soil, 2 water),  $K_2HPO_4$ -1 gm.,  $NaNO_3$ -1 gm., glycerol 10 gm. and agar 10 gm., pH 7.0). The crystal violet inhibits the growth of actinomycetes and gram-positive bacteria, but not the gram-negative bacteria. Radiobacter colonies are raised, smooth, glistening, with opaque center and transparent edge. Most of the strains brown milk. Growth of legumes increases the number of *B. radiobacter* in soil. It is found in greatest numbers near the plant, and often none at 1 foot distance from plant. Cowpeas, field peas, vetch and soybeans stimulate these bacteria in order named.<sup>26</sup> The increase in bacterial numbers is accompanied by the consumption of nitrates, not absorbed by the growing plants, and by an increase in the evolution of carbon dioxide.

A particular plant continuously grown in a soil leaves residues which will occasion a change in the chemical composition of the soil; these in turn influence the bacterial flora. Certain plant species favor certain types of bacteria and inhibit others, and thus disturb the bacterial equilibrium in the soil. The new flora thus established produces a specific change in the composition of the soil which affect subsequent plant growth, favoring some plant species and retarding others. This is true not only in case of favorable organisms, but also of plant pathogens; the continuous growth of a single plant, such as wheat, flax, clover, etc., will bring about the development of fungi pathogenic to this plant, making the soil "sick" for the particular plant.

Starkey<sup>27</sup> found that higher plants may affect certain groups of organisms to a different degree than others, and that the extent of the influence of various plants upon different organisms may not be the same. The greatest increase appears in the *B. radiobacter* group of organisms, although very striking effects are apparent in the general bacterial population. Potatoes increased the numbers only slightly while rape produced striking changes. The effect of the influence of any one plant upon the soil population is different at different stages

<sup>26</sup> Smith, N. R. Jour. Bact., 15: 20-21. 1928; Gräf, G. Centrbl. Bakt. II, 82: 44-69. 1930. For the distribution of *Azotobacter* in the root system of plants, see Poschenrieder, H. Centrbl. Bakt. II, 80: 369-378. 1930.

<sup>27</sup> Starkey, R. L. Soil Sci., 27: 319-334, 355-378, 433-444. 1929.

of growth (table 105). Slight effects are observed in the early stages of growth, maximum effects when plants reach considerable size and less upon the death of the plants. Due to their longer growing period, biennials show a much more prolonged effect upon the soil organisms than annuals. Higher plants may be a major factor in bringing about the so-called seasonal fluctuations of microorganisms, where tempera-

TABLE 105

*Influence of development of higher plants upon abundance of B. radiobacter*

PLANT	AGE OF DEVELOPMENT (DAYS)					
	44	63	86	138	173	Average of all periods
Fallow.....	540,000	920,000	900,000	320,000	420,000	620,000
Oats.....	780,000	7,800,000	6,320,000	860,000	670,000	3,290,000
Corn.....	680,000	2,020,000	3,180,000	3,340,000	960,000	2,040,000
Beans.....	1,980,000	2,540,000	4,400,000	360,000	1,640,000	2,180,000
Potatoes.....	780,000	980,000	5,340,000	1,200,000	500,000	1,760,000
Table beets.....	840,000	1,540,000	3,560,000	2,180,000	1,380,000	1,900,000
Mangel beets.....	1,400,000	6,400,000	3,160,000	4,000,000	1,400,000	3,270,000
Rape.....	46,600,000	8,600,000	6,360,000	5,120,000	3,640,000	14,060,000
Sweet clover*.....	1,140,000	2,000,000	1,900,000	120,000	2,820,000	1,700,000

\* For the sweet clover, the sampling periods are 25, 44, 67, 119, and 154 days

TABLE 106

*Influence of plants upon the production of nitrate in the soil*  
Parts per million

1908	FALLOW LAND	MAIZE	1909	FALLOW LAND	OATS
May 19.....	4.9	3.9	April 22.....	19.0	10.9
June 22.....	10.9	9.3	June 24.....	12.6	2.5
July 6.....	14.5	14.2	July 12.....	12.5	1.0
July 27.....	42.1	43.2	August 7.....	18.4	0.8
August 10.....	40.3	37.3			

ture and moisture do not appear to be related, as well as in the unequal distribution of microorganisms in soil.

*Influence of growing plants on nitrification in soil.* Lawes, Gilbert and Warington<sup>28</sup> were among the first to note that nitrogen of unmanured

<sup>28</sup> Lawes, J. B., Gilbert, J. H. and Warington, R. Jour. Roy. Agr. Soc., 19: 331-367. 1883.

land nitrifies with greater difficulty than nitrogen of land that has yielded large crops. More than twice as much nitrate nitrogen was formed, including the nitrogen in the crop and soil nitrate, in the plot growing oats as in the corresponding bare plot.<sup>29</sup> Certain plants, like maize, may, during the most active periods of growth, stimulate the formation of nitrates; during the latter periods of growth, when the roots cease to grow and begin to undergo decomposition, the same plants may exert a depressing effect (table 106).<sup>30</sup> This depressive influence was later

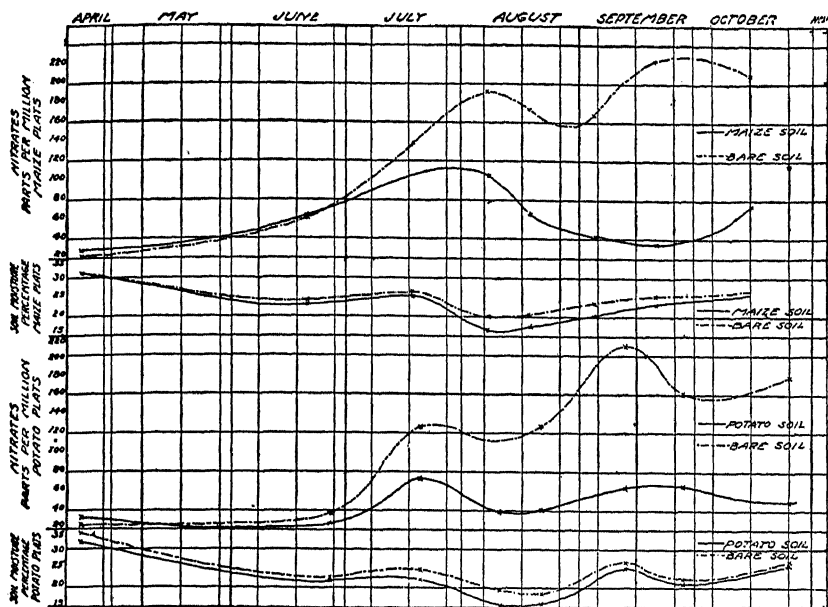


FIG. 79. Influence of crop upon nitrate content of the soil (after Lyon and Bizzell).

explained<sup>31</sup> by the fact that plant secretions and root residues are sources of energy for the soil organisms. The nature of the crop is of importance in this connection, due to the difference between the absorptive power of the plant and the carbon-nitrogen ratio of the plant residues.

<sup>29</sup> King, F. H. and Whitson, A. R. Wis. Agr. Exp. Sta. Bul., 93. 1902.

<sup>30</sup> Lyon, T. L. and Bizzell, J. A. N. Y. (Cornell) Univ. Agr. Exp. Sta. Mem.,

1. 1913; Jour. Frankl. Inst., 171: 1-16. 1911.

<sup>31</sup> Lyon, Bizzell and Wilson, 1923 (p. 614).

Nitrate production was found<sup>32</sup> to be higher under a cultivated crop, such as corn and potatoes (fig. 79), than under an uncultivated crop, such as wheat, rye, or timothy; this is particularly true of soils rich in organic matter, and may be due to the more frequent cultivation of the soil, on which the former crops are grown. An alfalfa or clover soil

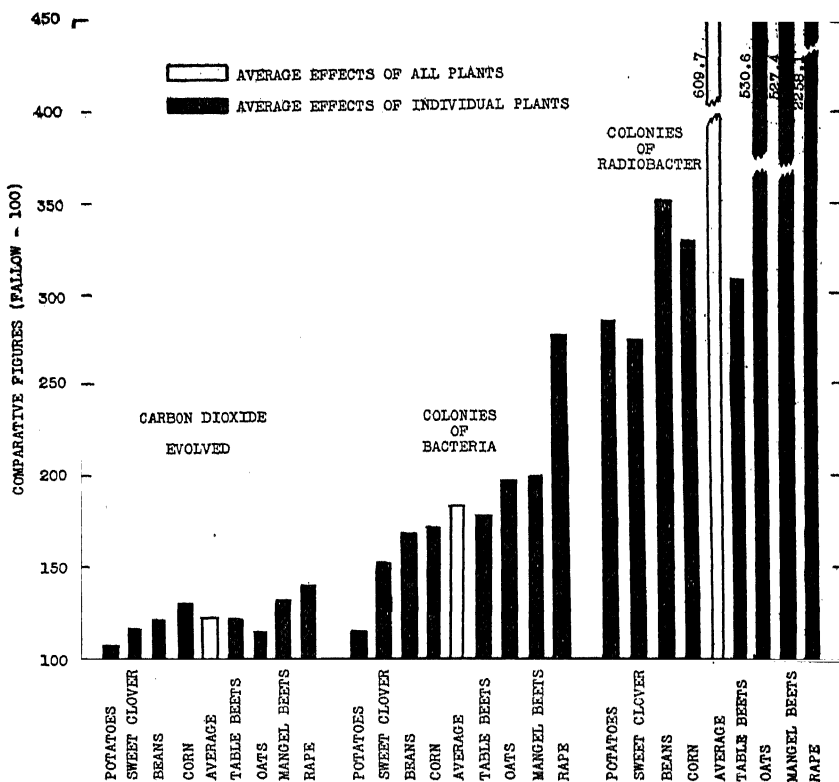


FIG. 80. Influence of plants upon the growth and activities of bacteria in soil (after Starkey).

kept fallow for two years was found<sup>33</sup> to nitrify dried blood more rapidly than a timothy soil similarly treated. McBeth and Smith<sup>34</sup> concluded

<sup>32</sup> Jensen, C. A. U. S. Dept. Agr. Bur. Pl. Ind. Bul., 173. 1910; Ladd, E. F. N. D. Agr. Exp. Sta. Bul. 47: 685-721. 1901; Lyon, T. L. and Bizzell, J. A. Soil Sci., 9: 53-54. 1920.

<sup>33</sup> Lyon, T. L. and Bizzell, J. A. Centrbl. Bakt. II, 37: 161-167. 1913.

<sup>34</sup> McBeth, I. G. and Smith, N. R. Centrbl. Bakt. II, 40: 24-51. 1914.

that the nitrifying power of a cropped and irrigated soil was higher than that of one which was not cropped. There was no increase in the nitrifying power of a semi arid soil, upon which legumes had previously grown; it was suggested that a growing crop may have a different influence upon nitrate formation in different soils.<sup>35</sup>

The growing crop may have a depressive effect upon the nitrate content of the soil (not necessarily nitrate-producing capacity of the soil).<sup>36</sup> It was suggested<sup>37</sup> that the lower nitrate formation in cropped land may be due to the adverse effect of the crop upon bacterial activities or to some process of destruction of nitrates, at work in the cropped soil which does not take place in the fallow soil.<sup>38</sup> The stimulative effect of the growing crop upon the activities of heterotrophic organisms (including decomposition of organic matter) and the injurious effect upon nitrate accumulation explain one another since one process is the cause of the other, the heterotrophic organisms, using the available energy of the fresh organic material of a wide carbon-nitrogen ratio, consume some of the nitrate.

*Influence of plants upon the evolution of carbon dioxide in soil.* The growing crop brings about an increase in the carbon dioxide content of the atmosphere, as indicated by the fact that the carbon dioxide content in a cropped soil is much greater than in a corresponding bare soil.<sup>39</sup> The excess of carbon dioxide was ascribed to the respiratory activity of the plants rather than to the decomposition of the root particles from the crop growing in the soil. This is in line with the previous observations of Barakov<sup>40</sup> that plants produce much greater quantities of carbon dioxide in the soil than do the bacteria; maximum carbon dioxide production was found to coincide with the maximum life activity of the plant. Leather<sup>41</sup> also found greater quantities of carbon dioxide in the neighborhood of roots of crops than in fallow land.

<sup>35</sup> Kellerman, K. F. and Wright, R. C. Jour. Amer. Soc. Agr., 6: 204-210. 1914.

<sup>36</sup> Voorhees, E. B., Lipman, J. G. and Brown, P. E. N. J. Agr. Exp. Sta. Bul., 210. 1907.

<sup>37</sup> Russell, E. J. Jour. Agr. Sci., 6: 18-57. 1914; 7: 1-45. 1915.

<sup>38</sup> Burd, J. S. Jour. Agr. Res., 12: 297-310. 1918.

<sup>39</sup> Russell and Appleyard, 1915 (p. 700); Turpin, H. W. Cornell Univ. Agr. Exp. Sta. Mem., 32: 319-362. 1920.

<sup>40</sup> Barakov, P. Zhur. Opit. Agron., 11: 321-342. 1910.

<sup>41</sup> Leather, J. W. Mem. Dept. Agr. India, Chem. Series, 4: 85-132. 1915; see also Bizzell, J. A. and Lyon, T. L. Jour. Amer. Soc. Agron., 10: 97-112. 1918.

Neller<sup>42</sup> obtained quantitative measurements of the total carbon dioxide liberated from oxidation processes taking place in the soil during plant growth; much more rapid oxidation was found to occur in a soil in which plants were growing than in the corresponding uncropped soil kept under the same conditions of moisture, aeration, temperature, etc. The growing roots were found to exert a direct influence upon the decomposition of organic matter in the soil. This will, of course, also bring about a greater liberation of available plant nutrients and thus stimulate further plant growth. A symbiotic relationship between the growing plant and the oxidizing organisms in the soil was, therefore, suggested. Further information on the influence of nature of crop upon the numbers of bacteria and evolution of CO<sub>2</sub> from soil is given in tables 107 and 108.<sup>42</sup>

TABLE 107

*Influence of plant upon the numbers of bacteria and evolution of CO<sub>2</sub>*

PLANT	BACTERIA PER 1 GRAM OF SOIL	SOIL REACTION	MILLIGRAMS OF CO <sub>2</sub> PRODUCED BY 1 KG. OF SOIL IN 24 HOURS AT 20°C.
	<i>millions</i>	<i>pH</i>	
Triticum vulgare.....	49	6.75	69.4
Secale cereale.....	42	6.44	68.2
Avena sativa.....	45	6.42	79.0
Beta vulgaris.....	78	6.89	74.3
Medicago sativa.....	120	6.89	86.8
Trifolium pratense.....	...	6.66	82.4

Starkey also found that the evolution of CO<sub>2</sub> was greater from soils which supported plant growth than in unplanted soil; however, the course of formation of the gas during the season was different for each of the plants. The influence of the plants on the formation of CO<sub>2</sub> was similar to the changes in the bacterial population in the soil: slight effect in the early stages of growth, greater effect with advance in vegetative development and fruiting, less subsequent to degeneration and death (fig. 80). Nitrification of the soil nitrogen was affected in a somewhat similar manner.

Lundegardh suggested that the fact that the roots of plants are sur-

<sup>42</sup> Neller, J. R. Soil Sci., 13: 139-159. 1922; Stoklasa, J. Chemie d. Zelle u. Gewebe, 12: 22-44. 1924.

rounded by a film of bacteria actively respiring explains to a large extent the formation of  $\text{CO}_2$  about the roots.<sup>43</sup>

The harmful effect of grass upon the growth of trees so commonly observed has been found<sup>44</sup> to be due to the fact that the surface roots of the trees are deprived of combined nitrogen; by producing a soil atmosphere rich in  $\text{CO}_2$ , the grass causes the surface roots to grow down and

TABLE 108

*Effect of buckwheat and of field peas upon the oxidation processes in a loam soil*

	INTER- VALS BE- TWEEN TITRA- TIONS	BUCKWHEAT		CHECK (NO PLANTS)		FIELD PEAS	
		Jar 1	Jar 2	Jar 3	Jar 4	Jar 5	Jar 6
	days	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
CO <sub>2</sub> withdrawn from system....	0-3	88.9	85.0	81.7	74.6	112.3	112.3
	4-5	23.9	15.8	46.5	34.2	55.1	47.6
	6-7	76.7	61.1	33.0	38.9	25.1	38.0
	8-10	39.7	6.5	32.0	21.7	37.3	33.7
	11-13	49.0	21.8	58.3	58.1	55.0	36.8
	14-18	79.6	40.0	41.4	46.8	7.9	10.5
	19-23	44.4	46.0	69.3	43.0	22.0	62.1
	24-32	27.0	20.1	121.0	79.7	22.1	15.8
Total.....		429.2	296.3	483.2	397.0	336.8	356.8
Dry weight of crop.....		786.8	842.8			1032.5	1024.6
Ash in crop.....		347.0	367.0			317.9	405.2
Organic matter in crop.....		439.8	475.8			714.6	619.4
Organic matter in seedlings.....		92.7	92.7			416.7	416.7
Organic matter produced during experiment.....		347.1	383.1			297.9	202.7
CO <sub>2</sub> fixed by plants during experiment.....		561.3	619.5			418.7	327.8
Total CO <sub>2</sub> obtained from soil....		990.5	915.5	483.2	397.0	818.5	684.6
Average CO <sub>2</sub> obtained from soil..		953.1 mgm.		440.1 mgm.		751.6 mgm.	
Average increase over check.....		116.5 per cent				70.8 per cent	

thus suffer from lack of oxygen; there was no evidence of the formation of a toxin by the grass.

*Influence of growing plants upon nitrogen-fixation in soil.* Heinze<sup>45</sup>

<sup>43</sup> Lundegardh, H. 1924 (p. 562).

<sup>44</sup> Howard, A. Proc. Roy. Soc., 97B: 284-321. 1925.

<sup>45</sup> Heinze, B. Landw. Jahrb. 39, *Erganzungbd.*, 3: 314-343. 1910.



found that fallowing increases the nitrogen-fixing capacity of the soil. Definite information on this problem is difficult to obtain since our methods are not sensitive enough to detect a minute increase in the total nitrogen of the soil. In most instances, the ideas presented concerning the stimulating effect of plants upon non-symbiotic nitrogen-fixation are largely speculative in nature. There is no doubt that plants excrete soluble substances through their roots which can be used as sources of energy by the bacteria. But whether this source is sufficient to increase appreciably the supply of fixed nitrogen in the soil remains to be determined.

In the case of leguminous plants, which offer a favorable substrate for the growth of different forms of *B. radicola*, the death of the plant and the decomposition of the roots lead to an increase in the numbers of the nodule organisms in the soil.

*Toxin production by plants.* The belief that plant roots excrete substances which are toxic to other plants of the same species existed since the early part of last century.<sup>46</sup> The favorable effect of plant rotation was believed to be based upon this principle. This idea persisted up until recent times, as shown by the contributions of Cameron, Whitney and others<sup>47</sup> who believed that soils contain toxins which originate largely from plant root excretions. According to Bolley,<sup>48</sup> however, the one-cropping system is responsible not for toxin accumulation, but for contamination with disease-producing microorganisms.

Frequently the injurious influence of plants upon microorganisms and *vice versa* are due to a competition for the available nitrogen and minerals in the soil. This phenomenon is referred to sometimes as a process of antagonism.<sup>49</sup> It can be overcome by the addition of available nutrients or partial sterilization of soil.

*Summary. The rhizosphere and the soil population.* Very little is known concerning the rôle of the rhizosphere, or the subterranean part of the plant system, in controlling this population.

Beijerinck<sup>50</sup> found larger numbers of *Azotobacter* in soil close to the roots of the leguminous plants than in soil from roots of non-legumes. The presence of greater numbers of bacteria, such as *Clostridium gelatinosum* and others, close to the plant roots as well as upon the root epi-

<sup>46</sup> DeCandolle. *Physiologie végétale.*, 3: 1474. 1832.

<sup>47</sup> U. S. Dept. Agr., Bur. Soils, Bul., 22, 23, 30, 36, 40, 47, 80. 1903-1911.

<sup>48</sup> Bolley, H. L. *Science*, N. S., 32: 529-541. 1910.

<sup>49</sup> Chouchack, 1927 (p. 763).

<sup>50</sup> Beijerinck, M. W. *Proc. K. Akad. Wetensch.*, 11: 67-74. 1908.

dermis than at a distance was reported by various investigators<sup>51</sup> for a number of plants, including beets, mangels, barley, etc. Wilson and Lyon<sup>52</sup> found that when sterilized soil was inoculated with soil supporting plant growth, much more abundant bacterial development took place than when unplanted soil was used for purposes of inoculation.

The sloughed off portions of the root caps, the root hairs, etc., may influence greatly the nature of the population developing in their neighborhood. The nature of the gases formed by the plants also influences the nature of the organisms developing in the particular locality. A certain soil decomposes cellulose with varying rapidity according to the kind of plants which have been growing in it; also, the nature of the organisms taking part in the decomposition of the cellulose varies with the plants grown in the soil.<sup>53</sup>

Whether we accept the concept of a rhizosphere or a specific bacterial population in the root zone of plants or not, it seems to be established beyond doubt that larger numbers of microorganisms find a more favorable condition for their development in the close proximity of plant roots than at a distance. Among these organisms, the development of nitrogen-fixing and cellulose decomposing bacteria is most marked; possibly the plants excrete or leave in the form of residues a certain amount of available energy, which would explain the development of the former; the cellulose-rich residues would naturally favor the development of cellulose-decomposing organisms in the soil. The specificity of these organisms will depend of course upon the nature of the residues and environmental conditions.

<sup>51</sup> Velich, A. *Ztschr. Zuckerind. Böhmen*, **27**: 975. 1903 (Centrbl. Bakt. II, 10: 678-679).

<sup>52</sup> Wilson, J. K. and Lyon, T. L. N. Y. (Cornell) *Agr. Exp. Sta. Mem.* 103. 1926.

<sup>53</sup> Rokitzkaia, A. *Jour. Sci. Inst. Amelior. Leningrad*, **13**: 168-208. 1926.

## CHAPTER XXXII

### SOIL AS A HABITAT FOR MICROORGANISMS CAUSING PLANT AND ANIMAL DISEASES

Microorganisms frequently compete with higher plants for specific soil nutrients; some of them may also produce substances which are directly injurious to higher plants. In addition to the injury which saprophytic microorganisms may cause to plants by their physiological processes, the soil also harbors organisms directly parasitic to plants or animals. In certain cases a soil condition may be brought about, commonly referred to as "sickness," which may not be a result of the activities of microorganisms directly pathogenic to plants, but which is due to certain processes brought about by saprophytic microorganisms.

*Saprophytism and parasitism among soil microorganisms.* Parasitism is a form of nutrition whereby an organism obtains its nutrients from the tissues or cells of another living organism. Symbiosis or mutual parasitism takes place when the host plant obtains nutrients from the invading organism. Parasitism is a matter of degree in the case of a number of organisms (especially fungi) found in the soil. (1) Some are strictly parasitic and are brought into the soil by the growing plant, by wind or other agencies. They cannot grow in the soil, but remain there alive and capable of causing the disease only for a short time; this is true of most smuts and certain bacteria among plant pathogens; *Bact. typhosum* is a case of an animal pathogen. (2) Some organisms continue to live in the soil for a season or more, but, in the absence of the host plant, they soon die out. They may, however, be able to attack a number of hosts and thus become more or less permanent in the soil, until all the host plants are completely removed; this is true of the club root organism of cruciferae, *Plasmodiophora brassicae*. (3) Some organisms are able to grow saprophytically in the soil for many years and become parasitic when the specific host plant is introduced. Various species of *Fusaria* (*F. oxysporum*, *F. radicicola*, *F. lini*, etc.), *Act. scabies*, certain *Rhizoctonia* (*Rh. solani*), and many other plant pathogens belong to this group. *Bac. tetani*, *Bac. anthracis*, *Bac. botulinus*, numerous gas gangrene types as *Bac. welchii*, and *Act. bovis* are instances of organisms capable of causing animal diseases, which may find a more or less per-

manent habitat in the soil. (4) Finally we find organisms primarily saprophytic in nature and abundantly distributed in the soil. They are capable, however, of causing diseases of the growing plant or various storage rots, when conditions become favorable. This group includes *Rh. nigricans*, certain species of *Fusarium*, *Penicillium*, etc.<sup>1</sup>

In the interaction of plants and microorganisms, various gradations between strict parasitism and strict symbiosis are found, with mutual parasitism as an intermediary phenomenon.<sup>2</sup> Mycorrhiza formation by fungi fills in the gap between parasitism and symbiosis. Some of the mycorrhiza are probably more symbiotic; others are often looked upon as parasitic in nature,<sup>3</sup> still others first attack the plant and then are digested by the plant juices. In the last instance we have a case of balanced parasitism; the root-tubercles of *Arbutus unedo* are caused by a fungus first ectotrophic, then endotrophic; the digestion of the fungus by the root tubercles confers immunity upon the root system as a whole.<sup>4</sup> On the other hand we find cases, like the infection of the thallus of the liverwort *Pellia epiphylla* with a species of *Phoma*,<sup>5</sup> where the fungus kills the protoplasmic contents of the infected cells. Since the plant can be grown without the fungus, the relationship is largely parasitic. The mycorrhiza of forest trees may be classed between these extremes.

The problem of host selection and host specialization has been studied carefully in the case of plant infesting nematodes.<sup>6</sup> The chief species of these organisms attack a large number of host plants. However, different populations of a nema species may prefer different host plants; when different host plants are growing in a given soil area the soil nemas will attack the one preferred, leaving the others unattacked even though they are favored host plants of the particular species. If peas or oats are grown on a soil for a number of successive years, *Heterodera schachtii* becomes so adapted to this host that it attacks this plant in the presence of a number of other plants, which may not be attacked at all. This observation was first made by Liebscher.<sup>7</sup> Nemas are capable of locating their host plants at considerable distances, moving even against the

<sup>1</sup> Strong, R. P. Science N. S., 61: 97-107. 1925.

<sup>2</sup> Bernard, N. Ann. Sci. Nat. Bot. 9 me Ser., 1909, 9; Caullery, M. Bull. Inst. Past., 19: 569-583, 617-627. 1921.

<sup>3</sup> Ciferri, R. Phytopath., 18: 249-262. 1928.

<sup>4</sup> Rivett, M. F. Ann. Bot., 38: 661-677. 1924.

<sup>5</sup> Ridler, W. F. F. Ann. Bot., 36: 193-208. 1922; 37: 483-488. 1923.

<sup>6</sup> Steiner, G. Phytopathol., 15: 499-534. 1925.

<sup>7</sup> Liebscher, G. Jour. Landw., 40: 357-368. 1892.

water-flow.<sup>8</sup> The nemas are capable of distinguishing closely related plant species. This was explained by the fact that although different species of plant-parasitic nemas feed on a wide range of host plants, a given population of one species will, if possible, always attack first the kind of host plants that its ancestors lived on. If this host plant is not available, host plants of near relationship (taxonomical, chemical) are sought and attacked. If the ancestors of a given population lived on a number of host plants for many generations, the population is polyphagous. If the ancestors of a population lived for many generations on a single species or variety of plant, their descendants will always attack that host plant and, only in exceptional cases, other plants, unless the host is absent; such a population is monophagous.

The growing plants seem to produce certain root secretions which are carried by the soil water and act as stimuli upon the nemas. The latter perceive the stimuli by means of a special sense organ (amphid). The nema then moves towards the points of higher concentration of the stimulating fluid until the host plant is reached.<sup>9</sup>

*Animal diseases caused by microorganisms found in the soil.* A number of bacteria capable of causing various animal diseases have been isolated from soil, where they find a natural habitat or persist only for longer or shorter periods of time. It is sufficient to mention that *Bac. anthracis* (Pasteur, Koch), *Bac. tetani* (Nicolai, Sanfelice), *Bac. chauvoei* (Arloing, Pellegrino), *Bact. pestis* (Jersin), *Bact. typhi*<sup>10</sup> (Macé) will persist in the soil for many months or even years. Pathogenic bacteria were often found to be actually capable of developing in the soil. Pasteur showed that earthworms can spread anthrax bacteria through the soil and even isolated the bacteria from the intestines of the worms. The soil may thus become a carrier of human disease, as demonstrated in the case of *V. cholerae* and *Bact. typhosum*.<sup>11</sup> *Bac. botulinus* is commonly found in soil; the presence of this organism has been demonstrated not only for infested soils but also for various virgin mountain and forest soils.<sup>12</sup>

<sup>8</sup> Baunacke, 1922 (p. 327).

<sup>9</sup> A detailed review of the relation between parasite and host plant, especially in respect to rusts, is given by Zimmermann. *Centrbl. Bakt.*, II, 65: 311-418. 1925.

<sup>10</sup> Kühler and Neufeld. *Ztschr. Hyg.*, 31: 133; Fischer, B. and Flatau. *Centrbl. Bakt.*, 29: 329. 1901.

<sup>11</sup> Prausnik, W. *Handb. der Hygiene* (Rubner usw.), 1: 520-562. 1911 (*Int. Mitt. Bodenk.*, 4: 239); Bail, O. and Breinl, F. *Arch. Hyg.*, 82: H. I. 1914.

<sup>12</sup> Meyer, K. F. and Dubovsky, B. J. *Jour. Inf. Dis.*, 31: 41-55. 1923; also 56-58, 59-94, 95-99, 100-109; Damon, S. R. and Payabal, L. B. *Ibid.*, 39: 491-501. 1926; Leighton, G. and Buxton, B. J. *Jour. Hyg.*, 28: 79-82. 1928.

*Bac. tetani* appears to be also universally distributed in the soil, especially in soils fertilized with animal manures and subject to the dust of the streets. Nicolaier<sup>13</sup> demonstrated the presence of this organism in over fifty per cent of the soils examined, an observation later confirmed by others. Out of 100 Scottish soils examined, four gave cultures producing botulinus toxin and eight tetanus toxin. It was even suggested<sup>14</sup> that the tetanus organism develops in rotting straw or manure, taking a part in processes of decomposition. The presence of this organism in the soil has also been ascribed to its presence in fecal excretions, due to its development in the intestine.

The subject of the gas gangrene producing bacteria has received special consideration in connection with the study of war wounds and trench fever. Spores of *Bac. sporogenes*, *Bac. welchii*, *Bac. tertius*, *Bac. oedematiens*, *Bac. bifermentans*, *Bac. cochlearius*, *Bac. tetani* and of other bacteria have been found<sup>15</sup> in all soils of Central Europe.

The nature of the soil, its physical, chemical and biological conditions have a marked influence upon the survival of these organisms in the soil. The bacterium causing fowl typhoid (*Bact. gallinarum*) will not remain in the soil for more than a week at a reaction of pH 6.2-6.4 or lower. However, at a pH of 6.7 to 7.0, the organism does not seem to be affected and will survive in the soil for at least 40 to 70 days. The organism causing white diarrhea in chickens (*Bact. pullorum*) shows somewhat greater susceptibility to acid soils than the *Bact. gallinarum*; it survived for over 64 days only in soils of pH 7.0. In moist soils, the organism was more viable and less susceptible to lower pH than in dry soils; it survived for eight days in soils of pH 6.2 to 7.0.<sup>16</sup> *B. tuberculosis* will survive in the soil for many years, without losing its virulence.<sup>17</sup>

There is no available information concerning the influence of the soil as an environment in modifying the activities of these pathogenic bacteria. It is known, however, that a common soil bacterium, *Bac. subtilis*, is capable of bringing about the destruction of the toxin of *Bac. botulinus*.<sup>18</sup> Very little is known concerning the soil as a medium for

<sup>13</sup> Nicolaier, A. Inaug. Diss. Göttingen. 1885; (Baumgart. Jahresber., 2: 270-272. 1886); see Lang, W. J. Schweiz. Arch. Tierheilk., 70: 249-265, 296-319. 1928; Leighton, G. and Buxton, J. B. Jour. Hyg., 28: 79-82. 1928.

<sup>14</sup> Vincent, H. Compt. Rend. Soc. Biol., 65: 12-14. 1908.

<sup>15</sup> Birger, J. Klin. Wochschr., 8: 598-599. 1929.

<sup>16</sup> Beaudette, F. R. Ann. Rept. N. J. Agr. Exp. Sta., 48 (1927): 254. 1928; 49 (1928): 293. 1929.

<sup>17</sup> Schottelius, M. Centrbl. Bakt., 7: 265-267. 1890.

<sup>18</sup> Stark, C. N., Sherman, J. M. and Stark, P. Proc. Soc. Exp. Biol. Med. 26: 343. 1929.

fungi and actinomyces (p. 288) causing human and animal diseases, although it has been established that forms like *Act. bovis*, various species of *Oidium*, *Monilia*, *Sporotrichum* and *Aspergillus*, capable of causing animal diseases (pulmonary infections, various skin diseases) are capable of finding a normal habitat in the soil.

*Bacteria causing plant diseases found in the soil.* The soil harbors various bacteria capable of causing plant diseases; these include *Bact. tumefaciens*, or the crown-gall organism, *Bact. campestris*, causing black-rot of cruciferae,<sup>18a</sup> as well as other important bacteria such as *Bact. solanacearum*, *Bac. phytophthorus*,<sup>19</sup> *Bact. phaseoli*, *Bact. tabacum*, and *Bact. beticolor*. *Bact. sepedonicum* causing bacterial rot of potatoes and a number of bacteria, like *Bac. mesentericus*, *Bac. carotovorus*, *B. aroideae* and others, pathogenic to potato tubers,<sup>20</sup> are also found to be able to persist in the soil. To the above bacteria, one may also add such organisms<sup>21</sup> as *Pseud. citriputeale*, *Bact. marginatum* and *Bac. atrosepticus*, capable of causing various plant diseases.

It has long been known<sup>22</sup> that the mosaic disease of tobacco is caused by a filterable virus; this has since been found to hold true for a large number of similar plant diseases, of the type known as infectious chlorosis. The nature of this virus is problematical. It has been held that in some cases the virus may persist in the soil, but this remains a question deserving further critical study. Tomato mosaic was found to be able to live for 4 to 6 weeks in field soils, but there was no evidence of overwintering of the virus.<sup>23</sup>

In the case of leguminous plants, the symbiotic association may change to one of parasitism, when conditions lead to a decrease in the carbohydrate formation by plants.<sup>24</sup>

*Plant diseases caused by fungi found in the soil.* Numerous fungi capable of causing plant diseases find their natural or temporary habitat in the soil. These fungi belong to the Myxomycetes (*Plasmodiophora brassicae* causing clubroot of cabbage), Phycomycetes (*Phytophthora infestans*, *Aphanomyces laevis*, *Synchytrium endobioticum*, *Pythium debaryanum*), Ascomycetes (*Botrytis cinerea*, *Sclerotinia trifoliorum*, *Cor-*

<sup>18a</sup> Clayton, E. E. N. Y. Agr. Exp. Sta. (Geneva), Bul., 506: 3-15. 1924.

<sup>19</sup> Smith, E. F. An introduction to bacterial diseases of plants. Sanders Co. Philadelphia, 1920.

<sup>20</sup> Brierley, P. Phytopathol., 18: 819-838. 1928.

<sup>21</sup> Patel, M. K. Phytopath., 3: 295-300. 1929.

<sup>22</sup> Beijerinck, M. W. Centrbl. Bakt., II, 5: 27. 1899.

<sup>23</sup> Doolittle, S. P. Phytopath., 18: 155. 1928.

<sup>24</sup> Thornton, H. G. Proc. Roy. Soc. London, B. 106: 110-122. 1930.

*ticism vagum*), Fungi Imperfecti (*Phoma betae*, *Verticillium alboatrum*, *Helminthosporium gramineum*, *Fusarium lini*, *Fusarium vasinfectum*), and finally certain Basidiomycetes, including smuts and others.

Various fungi have been isolated not only from cultivated soils where they might have been introduced, but also from virgin soils or from soils on which the particular host plant has never been grown before. Fungi, like *F. radicola* and *Rhizoctonia solani*, known to be parasitic on the Irish potato, were isolated from Idaho soils never cropped with potatoes, as well as from virgin desert lands.<sup>25</sup> Disease-free seed planted on new lands yielded a diseased product. Land previously planted to alfalfa, clover or grain is better adapted to the production of disease-free potatoes than virgin land. Some plant pathogenic fungi will persist in soil for many years, so that flax must be grown only on new soils. Various species of *Phytophthora* will also persist in soil for considerable periods of time and can withstand the low winter temperatures without much injury; they can also resist some desiccation. *Ph. infestans* can live saprophytically in soil, growing on old, partially decomposed plants. The pathogenicity of these fungi is not diminished by living in the soil.<sup>26</sup> Many plants are infected by fungi, the spores of which may not live in the soil but adhere to the seeds and produce a mycelium, which, on the germination of the seed, will attack the seedlings.<sup>27</sup>

Many of the fungi enumerated above are facultative parasites; in other words, they are capable of growing in soil in the absence of the host plant. The spores of *Sclerotinia trifoliorum*, for example, were found<sup>28</sup> to give rise to a mycelium which is at first saprophytic and then becomes facultative parasitic. The spores appear to germinate on vegetable residues in the soil; the mycelium spreads over the soil at a rate which depends on the environmental conditions. *Aphanomyces*, causing root rot of peas, develops in several stages, the zoospore stage being the infecting stage, the mycelium formation the spreading stage, and the oogonium formation the resting stage.

On the basis of the nature of the disease produced, the plant pathogenic fungi can be divided into several distinct groups:

1. Various damping off fungi, including *Pythium debaryanum*, *Sclerotinia*

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<sup>25</sup> Pratt, O. A. Jour. Agr. Res., 13: 73-100. 1918.

<sup>26</sup> Bruyn, H. L. G. de. Medd. Landbou. Wageningen., 24. 1922; Phytopath., 16: 121-145. 1926.

<sup>27</sup> Muth, F. Centrbl. Bakt. II, 21: 552. 1908.

<sup>28</sup> Wadham, S. M. New Phytol., 24: 50-56. 1925.



*libertiana*, *Phoma betae*, *Sclerotium rolfsii*,<sup>29</sup> *Rhizoctonia solani* (*Corticium vagum*), *Rhizoctonia* causing the damping off of conifers,<sup>30</sup> species of *Fusarium* and *Colletotrichum*.<sup>31</sup>

2. Root rots and other root infections, comprising a number of fungi, such as certain species of *Rhizoctonia*.<sup>32</sup> The constant culture of wheat on the same soil will bring about a condition of "wheat sickness." This is not a question of soil infertility or the formation of toxins detrimental to wheat, but is due to the introduction of fungi which cause various wheat diseases, by blighting, rotting and destroying the roots. These fungi are capable of persisting in the soil, living on the decomposing straw. In this group are found species of *Macrosporium*, *Alternaria*, *Helminthosporium*, *Fusarium* and *Colletotrichum*. *Asterocystis radialis*, a normal soil inhabiting organism, also attacks the roots of cereals. Although this fungus may in itself not cause any significant damage to crops, except oats, it may pave the way for the more vigorous parasites and thus contribute to the root-rot complex.<sup>33</sup>

Among the other specific root rots, one may include *Thielavia basicola* causing root rot of tobacco, legumes and many other plants; *Aphanomyces laevis*<sup>34</sup> causing root rot of beets; *Ozonium omnivorum* producing root rot of cotton and alfalfa. The root rots of peas are largely caused by four fungi, namely *Fusarium martii* var. *pisi*, *Pythium debaryanum*, *Corticium vagum* and *Aphanomyces euteiches*.<sup>35</sup> The cotton root rot, *Phymatotrichum omnivorum*, is capable of living in the soil for a number of years in the absence of the host plant.<sup>36</sup>

3. Wilts. *Fusarium oxysporum* has been isolated as a soil saprophyte.<sup>37</sup> This organism may cause a potato wilt disease. *F. lycopersicum* can also live as a saprophyte in the soil, upon the dead stems of the wilted tomato plants and on the soil organic matter; it can live in the soil several years retaining its virulence, even without the host plant.<sup>38</sup> The same is true of *F. conglutinans* and *F. lini*. Flax-sick soils are found<sup>39</sup> to contain *Colletotrichum lini*, *Polyspora lini*, *Fusarium lini* and *Thielavia basicola*. *F. hyperoxysporum* and *F. batatatis*, causing the stem-rot of sweet potato, may be generally disseminated in the soil; also other pathogenic Fusaria.<sup>40</sup> *Fusarium niveum* causing watermelon wilt may enter the

<sup>29</sup> Higgins, B. B. *Phytopath.* 17: 417-448. 1927; Rosen, M. R. and Shaw, L. *Jour. Agr. Res.* 39: 41-62. 1929.

<sup>30</sup> Wiant, J. S. *Cornell Univ. Exp. Sta. Mem.* 124. 1929; Toumey, J. W. and Li, T. T. *Yale Univ. School Forestr. Bul.*, 10. 1929.

<sup>31</sup> Hartley, C. *U. S. Dept. Agr. Bul.*, 934. 1921.

<sup>32</sup> Bolley, H. L. *N. D. Agr. Exp. Sta. Bul.*, 107. 1913; Beckwith, T. D. *Phytopathol.*, 1: 169. 1911; Braun, H. *Monogr. zum Pflanzensch.*, 5. 1930.

<sup>33</sup> Vanderpool, T. C. *Phytopath.*, 20: 677-680. 1930.

<sup>34</sup> Drechsler, C. *Jour. Agr. Res.*, 38: 309-361. 1929.

<sup>35</sup> Jones, F. R. *Jour. Agr. Res.*, 26: 459-476. 1923.

<sup>36</sup> Neal, D. C. *Science*, 70: 409-410. 1929; Ratliffe, G. T. *U. S. Dept. Agr. Circ.*, 67. 1929.

<sup>37</sup> Goss, R. W. *Nebraska Agr. Exp. Sta. Res. Bul.*, 23. 1923.

<sup>38</sup> Scott, 1924 (p. 789).

<sup>39</sup> Kletschetoff, A. *Jour. Timiriaseff Acad. Sci.*, 5: 69-81. 1930.

<sup>40</sup> Harter, L. L. and Field, E. C. *Phytopathol.*, 4: 279-304. 1914.

host through root hairs, root injuries and the epidermis of the hypocotyl; infested soils will bring about ready plant infection.<sup>41</sup> Among the wilt-producing fungi, we find also various species of *Verticillium*, which are known as a group to be typical soil organisms.

Among the other plant pathogenic fungi which can find their habitat in the soil, we may include different species of Rhizoctonia.<sup>42</sup> *Rh. solani*, for example, is abundant in cultivated land, where it lives on dead organic matter in the soil. When a proper host is introduced, the organisms may become active parasites, as in the damping-off of carnation cuttings, stem-rot and potato diseases; they can also attack a variety of weeds. *Rh. solani* attacks as many as 165 species of plants. *Spongospora subterranea* causes powdery scab of potatoes. *Synchytrium endobioticum* produces the wart disease of the potato and its spores may remain in the soil for two to eight years.<sup>43</sup> *Urophlyctis alfalfae* produces swellings on the roots of alfalfa. *Pythiacystis citrophthora* causes the brown rot of the lemon. *Sclerotium rolfsii* can propagate itself by mycelium in the soil, forming sclerotia under unfavorable conditions. *Rosellinia necatrix* and *Melanospora*<sup>44</sup> must also be mentioned. *Cercospora personata* is capable of multiplying in the soil saprophytically, preserving its virulence for eleven years.<sup>45</sup> Various smuts are often found in the soil and may persist there for long periods of time. *Urocystis tritici*, causing flag-smut of wheat, will survive in the soil; the spores germinate about the same time as the grain and infect the plant; in Australia, this is a most important disease. However, the extent to which rusts may persist in the soil has not been established yet.<sup>46</sup>

Cabbage and tomato sick soils may show as many as forty thousand colonies (on plate) of the parasitic organisms per gram of soil.<sup>47</sup> On land showing much root rot of corn, *Fusarium moniliforme* and a *Cephalosporium* have frequently been found. *Trichoderma koningi* and *Tr. lignorum*, two of the most common saprophytic soil fungi, are the causes of storage rots of sweet potato; the former is also associated with the so-called "ring rot."<sup>48</sup> The common soil organism *Rhizopus nigricans* is the cause of soft rot of sweet potatoes.

Some plants may be subject to attack by a great many organisms which find a permanent or only temporary existence in the soil. It is sufficient to cite<sup>49</sup> the diseases of the sweet potato found in the soil:

<sup>41</sup> Porter, D. R. Iowa Agr. Exp. Sta. Res. Bul., 112. 1928.

<sup>42</sup> Peltier, G. L. Ill. Agr. Exp. Sta. Bul., 189. 1916.

<sup>43</sup> Schander and Richter. Centrbl. Bakt., II, 58: 454-461. 1923.

<sup>44</sup> Delacroix and Maublanc. Maladies parasitaires des plantes cultivées. Bailliere Ed.

<sup>45</sup> Miège, E. Le désinfection du sol. Paris. 1918.

<sup>46</sup> Klebahn, cited by Waget, P. Rev. Prod. Chim., No. 22. 1920, 655; No. 4. 1921, 115; No. 6, 183.

<sup>47</sup> Manns, T. F. Del. Agr. Exp. Sta. Bul. 133, 35-36. 1922.

<sup>48</sup> Cook, M. T. and Taubenhaus, J. J. Phytopath., 1: 184-189. 1911.

<sup>49</sup> Harter, L. L. and Weimer, J. L. Tech. Bul. 99, U. S. Dept. Agr. 1929.

Stem rot, yellows or wilt caused by *Fusarium batatatis* and *F. hyperoxysporum*.

Black rot caused by *Ceratostomella fimbriata*.

Foot rot caused by *Plenodomus destruens*.

Texas root rot by *Phymatotrichum omnivorum*.

Scurf by *Monilochaetes infuscans*.

Soil rot (pox) by *Actinomyces poolensis*.

Mottle necrosis by *Pythium ultimum* and *P. scleroteichum*.

Sclerotial blight by *Sclerotium rolfsii*.

Rhizoctonia rot by *Corticium vagum* (*Rhizoctonia solani*), and various others.

Root knot caused by the nematode *Caconema radiculicola*.

Many of the infections found on sweet potatoes in storage may have their origin in the soil; this may be true of species of *Rhizopus*, *Mucor*, *Trichoderma*, *Fusarium*, *Alternaria*, etc.

The sugar cane may be attacked<sup>50</sup> by species of *Pythium*, *Rhizoctonia*, *Melanconium*, as well as numerous root-eating insects and worms. The disease may be a direct result of soil exhaustion and lack of proper fertilization or lack of sufficient oxygen supply.

*Plant and animal diseases caused by species of actinomyces.* Several plant pathogenic actinomyces have been found in the soil, including *Act. scabies*, the organism causing the common scab disease of potatoes and sugar beet.

On comparing a large number of actinomyces strains isolated from scabby potatoes, one can readily recognize that we are dealing here not with one species, but with a whole group which can be readily subdivided into several sub-groups, not only on the basis of physiological characteristics, but also on the basis of morphology. As a matter of fact, as many as 30 species of actinomyces have been described,<sup>51</sup> which are supposed to be causative agents of potato scab, the type of lesion being influenced by the species. The existence of more than one type of actinomyces capable of causing mangel-beet scab has also been suggested.

The formation of "pox" on sweet potatoes may be due, to some extent at least, to an actinomyces<sup>52</sup> found in the soil. The early results of Taubenhaus were confirmed by Manns who found<sup>53</sup> that the *Act. poolensis* kept in culture for many years caused the same type of "pox" as the recently isolated cultures of actinomyces.

<sup>50</sup> Matz, J. Jour. Dept. Agr. Porto Rico, 4: 28-40. 1920; Johnson and Stevenson. Ibid., 1, No. 4, 1917.

<sup>51</sup> Wollenweber, H. W. Der Kartoffelschorf. Arb. Forsch. Inst. Kartoffelbau. H. 2. 1920; Millard, W. A. and Burr, S. Ann. Appl. Biol., 13: 580-644. 1926.

<sup>52</sup> Taubenhaus, J. J. Jour. Agr. Res., 13: 437-450. 1918; Adams, J. F. Phytopath., 19: 179-190. 1929.

<sup>53</sup> Manns, T. F. Del. Agr. Exp. Sta. Rpt. 1925.

The causative agents of human and animal actinomycotic diseases are often claimed to be brought about by soil organisms or forms harbored upon plants.<sup>54</sup> Klinger<sup>55</sup> called attention to the fact that the aerobic actinomycetes commonly found on grasses and in straw infusions (also in soil) have never been isolated by him in any actinomycotic case. Only anaerobic forms were obtained from the latter; these develop on most media at temperatures above 30°C., and only seldom were cultures obtained which make a scant growth under aerobic conditions. Mixed infections consisting of anaerobes growing at body temperature together with aerobes are often obtained. We have to do here with species which have adapted themselves to a symbiosis with warm blooded animals, and which have almost nothing in common with aerobic saprophytes. However, there is no doubt that some aerobic actinomycetes are capable of causing infections of men and animals.

*Plant and animal diseases caused by invertebrate animals found in the soil.* Among the plant and animal pests present in the soil, we find protozoa, nematodes, wireworms, crustaceans, myriapods and insects. The plant parasitic nematodes include *Heterodera schachtii*, causing the disease of mangels; *Tylenchus tritici*<sup>56</sup> on wheat; *Heterodera radicola*, causing swellings or knots on roots of tomatoes, cucumbers, etc.,<sup>57</sup> *Tylenchus dipsae* (syn. *devastatrix*) causing the root knot on oats, tulip root, clover (one form of clover sickness); *Aphelenchus olesistus* causing leaf blight; *Tylenchus dipsaci* capable of causing galls on stems, leaves and tubers of potato, as well as many other plants which are susceptible to attack by this organism.<sup>58</sup>

The plant parasitic Gastropoda include *Agriolimax agrestis*. Among the Insecta one finds a number of Coleoptera, Lepidoptera, Diptera, etc. causing injury to plants. Wireworms also cause frequently considerable damage to crops, as when old meadows are plowed under and planted to corn or potatoes; they are capable of traveling considerable distances below the surface of the soil.<sup>59</sup> The nematodes and other worms, as well as the various insect pests are favored by the addition of organic matter. The soil as an environmental factor has also a considerable

<sup>54</sup> Odermatt, W. Schweiz. Med. Wochenschr., 50: 26-28. 1920.

<sup>55</sup> Klinger, R. Centrbl. Bakt., I, Or., 85: 357-359. 1921.

<sup>56</sup> Guenard, C. Zoologie agricole et Entomologie et Parasitologie agricole. Baillière Ed.

<sup>57</sup> Bessey, E. U. S. Dept. Agr., Bur. Pl. Ind. Bul., 217. 1911.

<sup>58</sup> Quanjer, H. M. Tijdschr. Plantenziekt., 33: 137-172. 1927.

<sup>59</sup> Hawkins, J. H. Maine Agr. Exp. Sta. Bul., 343. 1928; Miles, H. W. and Petherbridge, F. A. Ann. Appl. Biol., 14: 359-387. 1927.

direct or indirect effect upon the development of the European corn borer<sup>60</sup> and other injurious insects.

It has been shown<sup>61</sup> that a positive association exists between the intensity of plant disease caused by *Heterodera schachtii* and the cyst content of the soil, in those cases where the disease was observed recently. It is probable that the nematode disease is due to an association between the fungus *Rhizoctonia solani* and *Het. schachtii*.

The hookworm disease, caused by *Ancylostoma duodenale* and *Necator americanus*, is primarily caused by soil pollution. The larvae were found to develop in the soil protected by vegetation up to six months. The physical, chemical and biological soil conditions have a very important influence upon the development of hookworm larvae from infected faeces and upon the continued life of these larvae in the soil. The larvae are found largely in the capillary film of moisture surrounding the soil particles.<sup>61a</sup>

There is also a possibility that the soil harbors organisms which are parasitic upon plant and animal parasites, as was shown<sup>62</sup> in the case of a nematode parasitic on the Japanese beetle, *Popillia japonica*, which spends a large part of its life cycle in the soil.

*Relation of soil environment to plant infection.* The soil environment, including temperature, moisture, reaction and oxygen supply, has an important controlling influence upon all plant parasites found in the soil, whether they are obligate parasites or whether they can also exist in the soil in a saprophytic state. These environmental factors may determine not only the geographical distribution of the disease, but also its seasonal severity.<sup>63</sup>

The case of onion smut illustrates the possible importance of a specific factor of environment in determining the possible geographical range of soil parasites. This smut is a persistent soil born fungus, *Urocystis cepulae*, which is each year distributed throughout the United States on

<sup>60</sup> Huber, L. L. et al. Ohio Agr. Exp. Sta. Bul., 429. 1928.

<sup>61</sup> Smith, A. M. and Prentice, E. G. Ann. Appl. Biol., 16: 324-339, 340-346. 1929.

<sup>61a</sup> Baermann, G. Genesk. Tijdschr. Nederl. Indie, 57: 579-673. 1917; Stall, N. R. Amer. Jour. Hyg. July Suppl., 3: 1-36. 1923; Payne, F. K. Ibid. 547-583; Augustine, D. L. and Smillie, W. G. Ibid., March Suppl., 6: 36-62. 1926; Rickard, E. R. and Kerr, J. A. Jour. Prev. Med. 1: 185-203. 1926; Chandler, A. C. Hookworm disease. Macmillan Co., New York. 1929.

<sup>62</sup> Glaser, R. W. and Fox, H. Science, 71: 16-17. 1930.

<sup>63</sup> Jones, L. R. Amer. Jour. Bot., 11: 601-609. 1924; Plant world, 20: 229-237. 1917; Trans. Wis. Acad. Sci., XX: 433. 1922.

smutty onion sets. It infects the seedling onions only at low temperatures, being totally inhibited at the higher soil temperatures, 28°C. or above. As a result of that, although established in all the northern onion districts where onion seed is planted in cool soil in spring, it is unknown in the southern states, Texas and Louisiana, where the seed is planted in the autumn when the soil temperature is so high as to inhibit infection.

Different soil born parasites are affected very differently by environmental factors. Thus high soil temperatures stimulate the development of the *Fusarium* "yellows" disease of the cabbage and low temperatures inhibit it. By contrast the *Thielavia* root rot of tobacco is checked in warm soils and is seriously injurious only in cool soils. Jones further points out this seasonal contrast by citing evidence from two successive summers of which the one, 1915, was very cool, with a mid-summer soil temperature averaging about 5°C. lower than that of the succeeding summer. In the cool summer the *Thielavia* root rot of tobacco was unusually severe whereas the cabbage remained relatively free from disease. The succeeding year with its warm mid-summer period brought disaster to the cabbage crop because of the yellows disease, whereas the tobacco was free from root rot even on old "tobacco sick" soils. In general high soil temperatures favor the vascular *Fusarium* diseases, including flax wilt, *F. lini*,<sup>64</sup> tomato wilt, *F. lycopersici*,<sup>65</sup> and cabbage yellows, *F. conglutinans*.<sup>66</sup> High temperature (25–27°) also favors *Sclerotium rolfsii* and certain other plant pathogenic fungi.

On the other hand, not only the onion smut as noted earlier, but certain grain smuts are favored by low soil temperatures; the stinking smut of wheat, *Tilletia tritici*, thrives at 9–12° according to some,<sup>67</sup> and even at 5° to 10°, according to others.<sup>68</sup> *Rhizoctonia solani*, which may attack potato, cotton, and many other plants, is aggressive only at relatively low temperatures (less than 38°C.).<sup>69</sup> Of the two fungi capable of causing tomato wilt or "sleepy disease," *Verticillium albo-atrum* operates only at a low soil temperature (21–23°), whereas *Fusarium* wilt as already noted is a high temperature disease (28–29°).<sup>70</sup>

<sup>64</sup> Tisdale, W. H. *Phytopathol.*, 6: 412. 1916.

<sup>65</sup> Scott, I. T. *Missouri Agr. Exp. Sta. Res. Bul.*, 64. 1924.

<sup>66</sup> Gilman, J. C. *Ann. Mo. Bot. Gard.*, 3: 25–84. 1916; Tisdale, W. B. *Jour. Agr. Res.*, 24: 55–86. 1923.

<sup>67</sup> Hungerford, C. W. *Phytopathol.*, 12: 337–352. 1922.

<sup>68</sup> Faris, J. A. *Mycologia*, 16: 259–282. 1924.

<sup>69</sup> Richards, B. L. *Jour. Agr. Res.*, 21: 459–482. 1921.

<sup>70</sup> Bewley, W. F. *Ann. Appl. Biol.*, 9: 116–134. 1922; *Jour. Ministry Agr., Great Britain*, 30: 430–457. 1923.

Soil moisture also exerts a potent influence on many plant parasites. As previously noted, Sanford found dry soils favorable and wet soils inhibitory to potato scab. More often soil fungi, especially of the "damping off" types, are favored by high moisture; wet soils, even to the saturation point, favor the club root parasite of cabbage, *Plasmiodiophora brassicae*.<sup>71</sup> *Spongospora subterranea* (powdery scab) develops best in periods of damp, rainy, and cloudy weather and is favored by poor drainage.<sup>72</sup>

Hungerford recorded that there is a definite relation between the amount of moisture in the soil at seeding time and the amount of bunt or stinking smut which occurs in the resulting crop of wheat; the drier the soil at seeding time the less will be the amount of infection. When the soil is moist and cultivated frequently, the spores of *Tilletia tritici* rapidly lose their power of infection. There is also a certain relation between the lack of oxygen in soil and the occurrence of root rots.<sup>73</sup>

Usually in all such cases more than one variable factor is concerned. In the case of both cabbage club root and potato scab, it has long been known that soil reaction influences their occurrence. The above cited investigations, as well as the fact that high soil temperature, 22° or above, favors potato scab,<sup>74</sup> all point to the conclusion that when these potential parasites are present in the soil the occurrence and severity of the disease must be interpreted as a resultant of several variable environmental factors operating simultaneously.

The amount of organic matter present in the soil influences plant infection, since it offers a source of energy for the saprophytic existence of the organisms. *Thielavia basicola* cannot infect the host plant in pure sand, but can do so in the presence of organic matter, which allows the mycelium to exist for some time.<sup>75</sup> Clay soils are more favorable to infestation than sandy soils.<sup>76</sup> The *Fusarium* causing the root rot of peas is not disseminated by the seed, but spreads through the soil and is especially favored by a high content of organic matter. *Ozonium omnivorum*, the cotton and alfalfa root rot, spreads through the soil radially with a growth similar to fairy rings; it is favored by heavy

<sup>71</sup> Monteith, J. Jour. Agr. Res., 28: 549-561. 1924.

<sup>72</sup> Melhus, J. E., Rosenbaum, J. and Schultz, E. S. Jour. Agr. Res., 7: 213-254. 1916.

<sup>73</sup> Roedenburg, J. W. M. Zuurst. Grond. Verb. Proefschr. Univ. Utrecht. 1927.

<sup>74</sup> McKinney, H. H. Jour. Agr. Res., 26: 195-218. 1923.

<sup>75</sup> Massee, C. E. Roy. Gard. Kew. Bul. Misc. Inform. No. 1. 1912, 44-52.

<sup>76</sup> Johnson, J. and Hardman, R. E. Jour. Agr. Res., 17: 44-52. 1919.

soils, humid weather and dense cover crops.<sup>77</sup> The addition of organic matter to soil also favors the development of nematodes and rainworms. In some instances, the application of organic matter has helped to check the spread of a disease, as in the case of the cotton rot.<sup>78</sup>

In addition to organic matter, the use of artificial fertilizers and the nature of the soil have a considerable influence on the development of different organisms causing plant diseases.<sup>79</sup> Among the various plant nutrients added to the soil, the nitrogen and phosphorus sources are of the greatest importance in this connection. Foremost among the effects of treatment of soil is that resulting from changes in reaction.

*Influence of reaction upon the growth of plant pathogenic organisms in soil.* The plant parasites that can find a habitat in the soil can be divided,<sup>80</sup> on the basis of their response to soil reaction, into four groups:

1. Those parasites which prefer an alkaline reaction of the soil; these are included in the *litrophile* group. Here belong *Pythium debaryanum*, *Moniliopsis*, *Ophiobolus graminis*, *Typhula graminum*, *Fusarium nivale*, *F. equiseti*.

2. Those parasites which prefer a neutral soil reaction, or the *mesantypiphile* group; these include *F. avenaceum*, *F. herbarum*, *F. aurantiacum*, *Thielavia basicola*, *Phoma betae*. The last organism was found<sup>81</sup> to be a serious danger to sugar beets grown in soils of pH 8.0 or more; the safety region is pH 5.3–7.8.

3. Those that prefer an acid reaction, or the *oxyphile* group; here belong *Plasmodiophora brassicae*, *Rhizoctonia violacea*, *Synchytrium endobioticum*, the last forming the transition form to group four.

4. Those organisms that have no limited reaction optimum, or the *astatic* group; these comprise *Rhiz. solani*, *Helminthosp. sativum*, *F. culmorum*, *F. polymorphum* and *Ophiobolus herpotrichus*.

Infection under natural conditions is preceded by the saprophytic development of the fungus in the soil and the fungus attacks the plant only after it has reached in the soil a certain stage of development. Temperature has an important influence upon the critical point of the pH scale. For example, at a pH of 5.6 or less, there is no black root rot

<sup>77</sup> Duggar, B. M. Ann. Mo. Bot. Gard., 3: 11–23. 1916; King, C. J. Jour. Agr. Res., 26: 504–418. 1923.

<sup>78</sup> King, C. J. and Loomis, H. F. Jour. Agr. Res., 39: 199–222, 641–676. 1929.

<sup>79</sup> Ehrenberg, P. Fühling's Landw. Ztg. 1917, 130–132; 401–412; Levine, M. Amer. Jour. Bot., 8: 507–525. 1921.

<sup>80</sup> Schaffnit, E. and Meyer-Hermann, K. Phytopathol. Ztschr., 2: 99–166. 1930.

<sup>81</sup> Gallagher, P. H. Jour. Dept. Agr. Dublin., 29: 61–81. 1929.



of tobacco (*Thielavia basicola*) at any temperature; injury takes place at pH 5.7 at 15°C., at pH 5.8–5.9 at 27°C., no injury at 30°C. even at pH 6.0–6.9.<sup>82</sup>

Some plant pathogenic organisms are readily affected by certain hydrogen-ion concentrations of the soil which are not injurious to the growth of the host plant, as in the case of potato scab and wheat scab.<sup>83</sup> The acid minimum and the alkali maximum of some of the plant pathogens permit the use of various methods of control. *Act. scabies* and most other actinomycetes, for example, do not thrive well at pH less

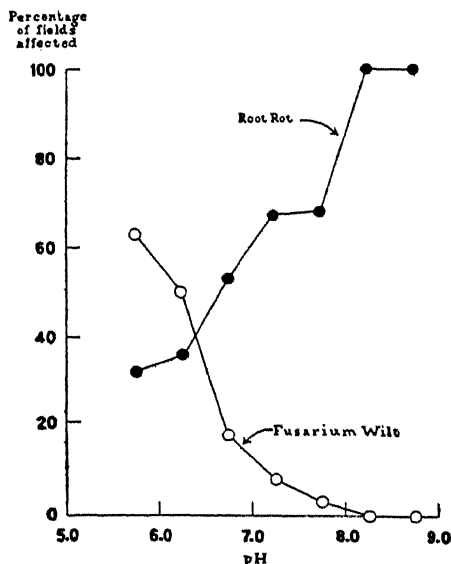


FIG. 81. Occurrence of root rot and Fusarium wilt with regard to the hydrogen ion concentration of the soil (from Taubenhaus et al.).

than 4.8; *Plasm. brassicae* is inhibited by an alkali reaction<sup>84</sup> obtained by the addition of lime. *Bact. solanacearum* causes serious infection in acid soils, but seldom in neutral or alkaline soils.<sup>85</sup> *F. lycopersici* causes minimum infection at pH 6.4 to 7.0; it has both an acid and an alkaline maximum.

<sup>82</sup> Doran, W. L. Jour. Agr. Res., 39: 853-872. 1929.

<sup>83</sup> Gillespie, L. J. Phytopathol., 8: 257-269. 1918; Soil Sci., 6: 219-236. 1918; Waksman, S. A. Soil Sci., 14: 61-79. 1922; Hopkins, R. F. Amer. Jour. Bot., 9: 159-179. 1922; McInnes, J. Phytopathol., 12: 290-294. 1922.

<sup>84</sup> Atkins, W. R. G. Sci. Proc. Roy. Soc. N. S., 16: 369-413. 1922.

<sup>85</sup> Arrhenius, O. Ark. Bot., 18: No. 1. 1922.

One type of plant may be subject to infection by certain fungi when the soil reaction is alkaline, while other fungi will attack the same plant under acid conditions. The fungus *Phymatotrichum omnivorum*, which causes the root rot of cotton is favored by a neutral or alkaline reaction; it is also more destructive in these soils than when found in acid soil. On the other hand, the Fusarium wilt of cotton (*F. vasinfectum*) is much more common in acid soils. This is brought out in fig. 81.<sup>86</sup> These facts must be taken into consideration in deciding upon the methods of control (fig. 82). Although the use of lime will tend to control certain

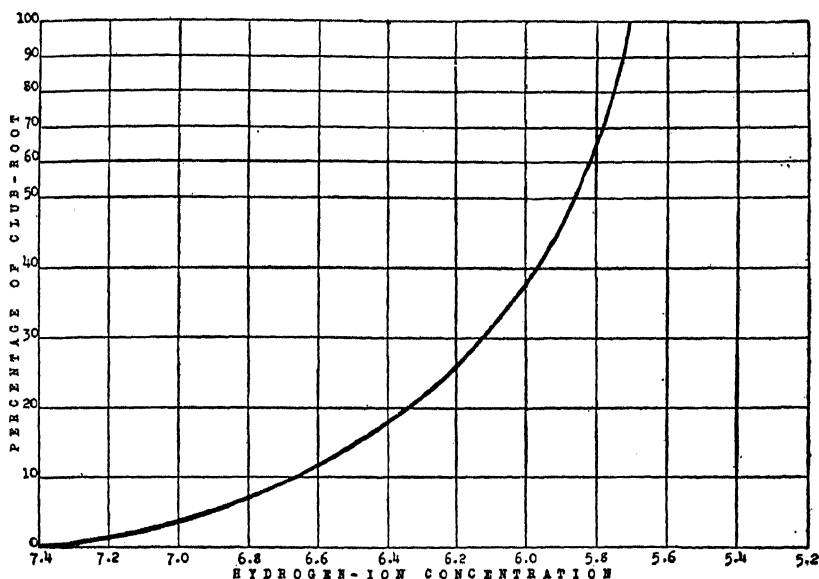


FIG. 82. The effect of soil acidity on the percentage of club-root (after Chupp.)

diseases of the beet, such as *Aphanomyces laevis*, it will favor at the same time the development of other diseases, such as the *Actinomyces* causing sugar-beet scab.

*Method of control.* To combat disease producing organisms, one has to know not only the life history of the pathogen, but frequently, as in the case of nemas, the life history of the host.

The methods of treatment of soil for the control of the injurious bacteria, fungi, nematodes, insects and the so-called "biological factors,"

<sup>86</sup> Taubenhaus, J. J., Ezekiel, N. N. and Killough, D. T. Texas Agr. Exp. Sta. Bul., 389. 1928.

such as "soil sickness," are divided into five distinct groups: 1. Proper rotation, or withholding of the host plant, since various parasites accumulate as a result of continuous growth of the same or closely related plants; use of resistant plant varieties, etc. 2. Special physical methods of soil treatment, such as soil cultivation, use of organic matter, specific fertilizers, etc. 3. Partial sterilization of soil, discussed in detail elsewhere (p. 723). 4. Use of chemicals for the destruction of specific organisms. 5. Biological control or introduction of organisms destructive to the parasites. In practicing crop rotation, one should remember the fact that many of the disease-producing organisms can persist in the soil for a number of years and some are actually capable of leading there a normal saprophytic existence. A rotation of at least five to six years should be practiced against the club-root of cruciferous plants and the sugar-beet nematode (*Heterodera schachtii*).

Partial sterilization of soil results not only in the destruction of harmful organisms, but also in increasing the fertility of the soil thus treated. The use of heat, formaldehyde, carbon bisulfide, coal-tar antiseptics, as well as certain inorganic substances (calcium sulfide, cyanides, and a mixture of copper sulfate and ammonium carbonate) come under this group of treatments.

The factors of soil aeration and soil drainage were found to be of considerable importance in favoring or checking certain diseases. Adequate drainage has been shown to be the most valuable method of control for the root rot of vine caused by *Clitocybe tabescens*.<sup>87</sup> In many cases the practice of flooding of soil to control the insects is utilized, as against Phylloxera in French vineyards or in the case of cranberry bogs to check certain insect attacks.

The methods of biological control of disease-producing organisms are still insufficiently studied. Here belong the introduction of birds and other higher animals, as well as of certain insects feeding upon certain injurious insects and worms, or the use of predacious nematodes against plant-pathogenic nematodes, or entomogenous fungi and bacteria parasitic upon insects.

The action of fertilizers in protecting the plant from disease consists in promoting the normal growth of the plant. However, the use of an excess of nitrogenous compounds may create a certain predisposition to disease, as in the case of potato to blight (*Phytophthora infestans*),<sup>88</sup> or

<sup>87</sup> Rhoads, A. S. Jour. Agr. Res., 30: 341-364. 1925; see Jones, F. R. and Drechsler, C. Ibid., 30: 293. 1925.

<sup>88</sup> Laurent, E. Exp. Sta. Rec., 11: 550. 1900.

to rust<sup>89</sup> and other diseases. A lack of available phosphorus and potash favors a decline in the plant's resistance to disease.<sup>90</sup> The morphological characters of the plant, its mode of growth, the chemical composition of the cell sap, especially its reaction, have been suggested as explanations of this phenomenon.<sup>91</sup> There are also exceptions to this rule. In the case of certain diseases, as the Verticillium wilt of tomato, the use of nitrogenous fertilizers proves to be favorable in increasing the resistance of the plant.<sup>92</sup>

For the control of various worms and insect larvae, the addition of phosphates, but no nitrogen, has been recommended. On the other hand, the addition of manure and of other organic fertilizers is recommended for reducing the damage done by root rots, wilts and certain other diseases caused by fungi.<sup>93</sup> Nematodes in sugar cane soils are controlled by the use of various organic substances, while CaO and phosphates are said to produce heavy infestation.<sup>94</sup>

A number of chemical compounds have been recommended for the control of various fungi and nematodes in the soil. The saturation of soil with formaldehyde to prevent spreading of disease-producing organisms has often been practiced. Formaldehyde in concentrations of 0.045 to 0.05 per cent was found to give very good results in combatting the sugar-beet nematodes; CS<sub>2</sub> is not effective even in large concentrations against nematodes. It is difficult to reach the nematodes at a depth lower than 60 cm., and it is difficult to have the poison penetrate the whole mass of soil.<sup>95</sup> The nematodes present in the lower depths of soil and in the form of cysts can be made to develop and come nearer the surface by the use of catch crops and chemical stimulants. In the absence of the host plant, the nematode larvae die off.<sup>96</sup> Formalin, sometimes following crude benzol treatment for control of potato wart,<sup>97</sup>

<sup>89</sup> Spinks, G. T. Jour. Agr. Sci., 5: 231. 1913.

<sup>90</sup> Stakman, E. C. and Aamodt, O. C. Jour. Agr. Res., 27: 341. 1924.

<sup>91</sup> Martin, H. The scientific principles of plant protection. Longmans, Green & Co. New York. 1928.

<sup>92</sup> Bewley, W. F. Diseases of glasshouse plants. London. 1923.

<sup>93</sup> Fulton, H. R. Science, 66: 193-194. 1927; La. Agr. Exp. Sta. Bul., 96. 1907; Orton. U. S. Dept. Agr. Farmer's Bul., 333. 1910; Rosen, Science, 65: 616-617. 1926; King and Loomis. Jour. Agr. Res., 32: 297-310. 1926.

<sup>94</sup> Swezey, O. H. Proc. Hawaiian Sugar Plant. Assn. 47th. Ann. Meet., 1927: 94-106. 1928.

<sup>95</sup> Mölz, E. Deut. landw. Presse, 53: 195-196. 1926.

<sup>96</sup> Baunacke and Reusch. Mitt. deut. landw. Gesell., 1924, 401.

<sup>97</sup> Korf, G. and Böning, K. Phytopath. Ztschr., 2: 39-86. 1930; Lemberzahl, J. Ibid., 2: 257-320. 1930.

mercury bichloride and other disinfectants are recommended for the control of various plant diseases.<sup>97</sup> The treatment of soil with a 1:1000 or 1:1,200 solution of mercuric chloride was found to be effective in controlling root maggot, black rot, club root and damping-off diseases.

Arsenic is employed to a considerable extent in China, by mixing with ashes for soil dressing, for the destruction of worms; a similar practice is used for golf greens. Acetic acid (1.2%), applied 10 days before planting, has also been recommended<sup>98</sup> for the destruction of soil infesting damping-off fungi (species of *Pythium*, *Rhizoctonia*).

Various other soil fungicides and volatile antiseptics, like CS<sub>2</sub> and toluol, have been frequently employed for the destruction of the pathogenic fungi.<sup>99</sup> Carbon bisulfide can be used with success against a number of fungi such as *Dematophora necatrix*, *Rhizoctonia* and *Synchytrium*. This disinfectant should be applied to the soil free from plants, otherwise the chemical will result in plant injury. Miège obtained the best results with toluol in controlling *Sclerotinia libertiana* and *Fusarium lycopersici*.<sup>100</sup>

It is important to remember in this connection, that the organic disinfectants will be decomposed in the soil by various groups of micro-organisms. A substance injurious to microbes may become stimulating as a result of its transformation in the soil. This is true of aniline, nitrobenzol and cyanamide.<sup>97</sup>

For the control of damping-off fungi, chemical treatment of soil has been found to be quite efficient.<sup>101</sup> The treatment with CuSO<sub>4</sub> has been recommended<sup>102</sup> for the control of root rots of wheat, such as *Ophiobolus graminis*, *Leptosphaeria herpotrichoides* and *Fusarium* sp. The damping-off diseases of conifers can be controlled<sup>103</sup> by soil treatment at time of seeding.

In addition to these chemical treatments, various other soil fumigants and disinfectants are employed, as potassium xanthate, calcium cyanamide, when applied in doses of 0.1 per cent.<sup>104</sup>

<sup>98</sup> Doran, W. L. Jour. Agr. Res., **36**: 269-280. 1928.

<sup>99</sup> Halsted, B. D. N. J. Agr. Exp. Sta., Sp. Bul. S., 1900; Johnson, J. Wis. Agr. Exp. Sta. Res. Bul., **31**, 29-61. 1914; Gingham, C. T., and Spinks, G. T. Jour. Bat. and West and South Cont. Soc., **14**: 126-130. 1920.

<sup>100</sup> Miège, 1917 (p. 740).

<sup>101</sup> Thomas, H. E. Phytopath., **17**: 499-506. 1927.

<sup>102</sup> Menecacci, M. Boll. R. Staz. Pat. Veg., **8**: 312-332. 1928.

<sup>103</sup> Wiant, J. S. Mem. 124, Cornell Univ. Agr. Exp. Sta., 1929.

<sup>104</sup> de Ong, E. R. Jour. Ind. Eng. Chem., **18**: 52. 1926; Peyronel, B. Bol. R. Staz. Pathol. Veg. Rome., **6**: 138-144. 1926.

When soil is steamed, the fungi are readily destroyed; but once a parasitic organism like *Pythium debaryanum* is introduced, it will readily develop in the treated soil and may even cause a larger amount of infection. This parasitic activity can be decreased by inoculating the treated soil with various saprophytic fungi. Treatment of soil with a disinfecting agent followed by inoculation with saprophytic fungi may prove to be most efficient in increasing the value of the treatment.<sup>105</sup> Whether the saprophytic fungus uses up the available nutrients rendered soluble on steaming of soil or whether this is due to the production of a substance directly injurious to the plant pathogen, remains to be determined. The possibility of the formation by various common soil bacteria and fungi of a complex, capable of passing through a Berkefeld filter, which will suppress the pathogenicity of *Ophiobolus graminis* causing cereal root rots has been indicated.<sup>105a</sup>

In order to destroy pathogenic fungi by heat, in the case of greenhouse soils, the following temperatures must be attained:<sup>106</sup>

INFECTING ORGANISM	TEMPERATURE OF CONTROL	
	18 hours	Few minutes
	°C.	°C.
Nematodes.....	40	60
<i>Pythium</i> .....		60
<i>Rhizoctonia</i> .....		80
<i>Sclerotinia</i> .....		80
<i>Septoria lycopersici</i> , spores.....		55
Anthraxnose beans, spores.....		48
Anthraxnose beans, mycelium.....		65
Corn root rot <sup>107</sup> .....		65

Among the most efficient methods of control of soil borne infections is the adjustment of the soil reaction, either by the use of alkali-forming (lime), or acid forming (sulfur, ammonium sulfate) materials. The addition of sulfur or inorganic acids to soils having a reaction of pH 5.9 or

<sup>105</sup> Hartley, C. U. S. Dept. Agr. Prof. Paper Bul., 934. 1921.

<sup>105a</sup> Sanford, G. B. and Broadfoot, W. C. Sci. Agr., 11: 512-528. 1931.

<sup>106</sup> Brown, H. D., Baldwin, I. L. and Conner, S. D. Purdue Univ. Agr. Exp. Sta. Bul., 226. 1922; see also Hunt, N. R., O'Donnell, F. G. and Marshall, R. P. Jour. Agr. Res., 31: 301-363. 1925; Beinhart, E. G. U. S. Dept. Agr. Farm. Bul., 996. 1918; Byers, L. P. and Gilbert, W. W. U. S. Dept. Agr. Bul., 818. 1920.

<sup>107</sup> Valleau, W. D., Karraker, P. E. and Johnson, E. M. Jour. Agr. Res., 33: 453-476. 1926.

above is recommended, the amount of sulfur or acid to be used depends of course on the initial reaction and buffer content of the soil.<sup>108</sup> However, the action of sulfur in controlling the wart disease of potatoes does not depend alone upon the acidity produced, but also upon some other mode of action of the sulfur,<sup>109</sup> probably thiosulfuric acid produced at an early stage of oxidation of the sulfur.

*Plasmodiophora brassicae*, the organism responsible for the clubroot of cabbage, can be controlled by the use of lime sufficient to raise the pH value of the soil above 7.0. The amount of disease rises very rapidly as soil acidity increases. At pH 6.0 or even above, 100 per cent of infection is possible<sup>110</sup> (fig. 82).

For the control of potato scab, sulfur is used with success. Care must be exercised, however, in using the proper amounts, so as not to make the soil too acid. Sweet potato scurf and pox of sweet potatoes can also be checked by the application of sulfur. Application of lime, which produces a favorable reaction, and of barnyard manure favor the development of scab. The addition of acid fertilizers (acid phosphate) or fertilizers which make the soil reaction acid (sulfur, ammonium salts) tends to decrease the development of scab, as shown elsewhere (p. 288). According to Millard,<sup>111</sup> sufficiently liberal dressings of green manure added to the soil will inhibit the disease; this is probably due to the temporary increase in soil acidity, as a result of the decomposition of the organic matter by the soil fungi, and to an increase in soil moisture; scab is much more prevalent in dry seasons, since actinomyces are much less active in very moist soils. Sanford suggested that the soil reaction may not be the important factor in controlling the development of potato scab in the soil. Moisture was found to be directly or indirectly the main factor, a high moisture content controlling the disease, while abundant scab is formed in dry soils. The development of scab is influenced also by the temperature of the soil, the optimum for scab being 22°C.<sup>112</sup>

<sup>108</sup> Anderson, P. J. A., Osmun, A. V. and Doran, W. L. Mass. Agr. Exp. Sta. Bul., 229. 1926; Science, N. S., 66: 661-662. 1927; Conn. Tobacco Sta. Bul., 8. 1927.

<sup>109</sup> Crowther, E. M., Glynne, M. D. and Roach, W. A. Ann. Appl. Biol., 14: 422-427. 1927; Roach, W. A. Jour. Agr. Sci., 20: 74-96. 1930.

<sup>110</sup> Chupp, C. Phytopathol., 18: 301-306. 1928.

<sup>111</sup> Millard, W. A. Ann. Appl. Biol., 9: 156-164. 1922; 10: 70-88. 1923; 14: 202-216. 1927.

<sup>112</sup> Sanford, G. B. Phytopath., 13: 231-236. 1923; Jones, L. R. et al. Wis. Agr. Exp. Sta. Bul., 53. 1922.

The treatment of certain physiological soil diseases has aroused recently considerable attention, especially in connection with the growth of plants on certain types of peat soils. The use of  $\text{CuSO}_4$ , in small doses, has been found to give best results in neutralizing the effect of the so-called peat sickness.<sup>113</sup> This disease is believed to be due to a certain toxic substance present in peat, which is soluble in alcohol; it is precipitated by copper and its injurious effect upon plants thus neutralized.<sup>114</sup>

<sup>113</sup> Hoodig, J., Meyer, C. and Goodyk. *Ztschr. Pflanz. Düng. Bodenk.*, 8A: 14-52. 1926; Allison, R. V., Bryan, O. C. and Hunter, J. H. *Florida Agr. Exp. Sta. Bul.*, 190. 1927.

<sup>114</sup> Smith, W. S. Thesis. Wageningen. 1927.



## CHAPTER XXXIII

### SOIL INOCULATION

*Beneficial and injurious microbiological processes in the soil.* The growth of higher cultivated plants is usually taken as a criterion in determining whether a certain microorganism or a certain microbiological process is beneficial or injurious. But a careful study of these processes and the organisms concerned can hardly justify such a strict division in all cases. Some, like the nitrogen-fixing, nitrifying and sulfur oxidizing bacteria, and various organisms decomposing celluloses and proteins, are no doubt beneficial to the growth of higher plants. Plant pathogenic fungi, like certain species of *Fusarium*, *Rhizoctonia* and *Pythium*, may, no doubt, become injurious, when environmental conditions are favorable. We may even call nitrate-reducing and sulfur reducing bacteria harmful, although their action is indirect and depends entirely upon soil conditions.

Some organisms may carry on processes in the soil which are both injurious and beneficial to the growth of higher plants; a certain process may be beneficial at one time and injurious at another. The question becomes one of mere relative importance. A fungus, like *Trichoderma* or *Asp. fumigatus*, decomposes cellulose rapidly and is no doubt beneficial, but it also synthesizes considerable protoplasm and stores away large amounts of nitrogen and it becomes, therefore, temporarily injurious to higher plants. When a protein is decomposed by fungi, smaller amounts of ammonia are liberated than when it is decomposed by bacteria. When the protein is added to the soil, an acid soil favors the development of fungi, while a neutral or alkaline soil favors the development of bacteria and actinomycetes.

Facts like these, as well as conditions which may favor the development of different groups of soil microorganisms and the presence or absence in the soil of a particular organism, must be known before we can make use of the principle of soil inoculation. This consists not merely in the introduction of useful organisms which may be lacking in the soil, but also in making soil conditions favorable for the biological processes useful to the growth of higher plants.

*Introduction of certain useful microorganisms into the soil.* Among

the useful microorganisms, which may have to be introduced into the soil are: (1) those which carry on important processes beneficial to a specific plant or to plant growth as a whole; (2) strains more vigorous than those already found in the soil; (3) organisms which destroy or injure the development of such microbes, which are directly harmful to higher plants. It is not merely sufficient to introduce the beneficial organisms, but the conditions should be made favorable for their development in the soil.

So far as our present knowledge of soil biological processes is concerned, the microorganisms which may be lacking in the soil, or whose activities in the soil are to be stimulated are as follows: (1) symbiotic and non-symbiotic nitrogen fixing bacteria; (2) sulfur-oxidizing bacteria; (3) nitrifying bacteria, and (4) microorganisms capable of vigorous decomposition of the soil organic matter. The favorable influence of small quantities of manure added to the soil has been frequently ascribed to the inoculating power of various bacteria present in the manure, these organisms presumably decomposing the soil organic matter more vigorously than the native flora. However, this favorable action is probably due not to the organisms introduced, but rather to the presence in the manure of certain inorganic substances, such as available nitrogen and phosphates, which stimulate the growth of higher plants or of certain soil organisms. Most of the bacteria capable of decomposing starch and cellulose in the intestinal tract of animals are specific inhabitants of the tract and are not found in great abundance outside of the animal.<sup>1</sup>

The common assumption that the presence of nodules on leguminous plants is sufficient proof that the soil does not have to be inoculated with the specific bacteria has been disproven by Fred and associates, who have differentiated between good and poor strains of nodule bacteria, the latter often actually becoming parasitic upon the plant.

Among the organisms which may directly destroy or otherwise eliminate the activities of soil inhabitants directly injurious to higher plants, the following may be mentioned: (1) the predacious nematodes, like *Mononchus*, which destroy the injurious nematodes, such as *Heterodera* or *Tylenchus*;<sup>2</sup> (2) saprophytic fungi which may act as a check to the development of pathogenic fungi.

Among the soil conditions, which may have to be modified, so as to stimulate the development of organisms whose activities in the soil are

<sup>1</sup> Henneberg, W. *Centrbl. Bakt.* II, 55: 242-281. 1922.

<sup>2</sup> Thorne, G. *Jour. Agr. Res.*, 34: 265-286. 1927.

favorable to the growth of higher plants, the following may be included: (1) a proper carbon-nitrogen ratio of the soil; (2) a favorable soil reaction and presence of sufficient bases; (3) presence of inorganic nutrients, especially phosphates and potassium salts; (4) soil moisture and aeration. In addition to these, certain specific treatments may prove to be useful for the control of specific microorganisms, as presence of available carbohydrates for the stimulation of non-symbiotic nitrogen fixation; a certain reaction, for the control of specific plant diseases; soil sterilization, for the improvement of the physical and chemical conditions of the soil and the elimination of certain injurious microorganisms.

When the soil is to be inoculated with certain microorganisms, one may choose between the use of (1) soil, in which the desired crop has been grown successfully; (2) pure cultures, or artificially prepared mixed cultures; (3) specific vigorous strains, which are more active than those already present in the soil.

*Legume inoculation.*<sup>3</sup> The first inoculation test on record is the experiment carried out in 1887 at the Bremen Experiment Station. It was found that a good stand of clover was obtained on newly drained heath or swamp soils inoculated with old soil in which clover was grown, provided the swamp soil was limed properly. In comparing the use of soil, in which the specific legume was grown, with pure cultures, for inoculation purposes, it is often found that soil gives better results. Hiltner<sup>4</sup> suggested that this may be due to the fact that, when the seed germinates, certain toxic substances pass out from the embryo which seem to be toxic to the bacteria; this danger can be obviated by inoculating the soil directly rather than the seed.

Atwater and Woods<sup>5</sup> were among the first in America to show the favorable effect of nodules on the growth and nitrogen content of alfalfa and peas. The gain in nitrogen was proportional to the number of nodules on the roots. Warington<sup>6</sup> soon demonstrated that the growth of properly inoculated legumes resulted in an increase in the nitrogen content of the soil; part of a wheat field seeded to clover contained 0.156 per cent nitrogen and only 0.142 per cent was found in the part of a field

<sup>3</sup> A detailed review of the earlier literature on legume inoculation is given by K. F. Kellerman. *Centrbl. Bakt.*, II, 34: 42-50. 1912; Löhnis, F. and Leonard, L. T. U. S. Dept. Agr. Farm. Bul., 1496. 1926.

<sup>4</sup> Hiltner, L. *Deut. landw. Presse*, 29: 15, 119. 1902.

<sup>5</sup> Atwater, W. O. and Woods, C. D. *Storrs Agr. Exp. Sta. Rpt.*, 2: 11-51, 1889-90.

<sup>6</sup> Warington, R. U. S. Dept. of Agr. Off. Exp. Sta. Bul., 8, 22-41. 1892.

seeded to barley. The nitrogen content of inoculated plants was found to be much higher than that of uninoculated plants, Nobbe and Richter<sup>7</sup> reporting 4.29 per cent of nitrogen for the first as against 1.85 per cent for the second. These results were soon confirmed by a number of workers in Europe and in America, as shown later.

In 1896 Nobbe<sup>8</sup> suggested the use of pure cultures of *B. radiculicola* for the inoculation of leguminous plants. A product placed on the market consisted at first of *B. radiculicola* grown on gelatin. However, gelatin was found to be an unsuitable medium for the growth of this organism. It was then replaced by a liquid medium, namely a 2 per cent peptone solution or skimmed milk,<sup>9</sup> and later by agar media. In 1902 the use of cotton cultures was introduced.<sup>10</sup> Cotton was placed in a liquid culture of bacteria, then dried and placed in packages. This was accompanied by two packages of nutrient substances, the first containing sugar,  $K_2HPO_4$  and  $MgSO_4$ , and the second ammonium phosphate. Before using, the cotton was placed in boiled water and the contents of the first package added. After 24 hours at 20°, the contents of the second package were added and the culture allowed to incubate; this culture was then used for moistening the seed or for inoculation of a small amount of soil which was then spread over the field. Usually mixtures of various legume bacteria were employed. The result proved to be unsatisfactory, due to the fact that the bacteria could not withstand the process of drying on cotton.<sup>11</sup>

Various other preparations, consisting of liquid, semiliquid or solid cultures were soon introduced. However, the early beneficial results secured with these pure cultures by Nobbe and others<sup>12</sup> were not confirmed by investigators both in America<sup>13</sup> and in Europe,<sup>14</sup> who found soil to be superior to artificial cultures for inoculation purpose. The inoculating value of some of these earlier preparations has been com-

<sup>7</sup> Nobbe, F. and Richter, L. Landw. Vers. Sta., 59: 167-174. 1903.

<sup>8</sup> Nobbe, F. Bot. Centrbl., 68: 171-173. 1896; Hiltner, L. Arb. K. Gesundheitsant. Biol. Abt., 1: 177-222. 1900.

<sup>9</sup> Hiltner, L. and Störmer, K. Arb. K. Gesundheitsant. Biol. Abt., 3: 151. 1903.

<sup>10</sup> Moore, G. T. U. S. Dept. Agr. Yearbook 1902-1903, 333-342; Bur. Pl. Ind. Bul., 71. 1905.

<sup>11</sup> Simon, J. Jahresb. Ver. Angew. Bot., 5: 132-160. 1907.

<sup>12</sup> Edwards, S. F. and Barlow, B. Ont. Dept. Agr. Bul., 164. 1908.

<sup>13</sup> Stevens, F. L. and Temple, J. C. N. C. Agr. Exp. Sta., 30th Ann. Rept., 48-57. 1908.

<sup>14</sup> Barthel, C. Meddel. Centralanst. Försöksv. Jordbruk., 95. 1914; K. Landbr. Akad. Handb. o. Tdskr., 53: 251-280. 1914.

pared critically with the inoculating value of soil on which the particular legumes were grown.<sup>15</sup> The reason for the failure to obtain in some instances results from artificial inoculation may have been due, in some cases at least, to the fact that fresh legume seeds usually have on them the nodule-forming bacteria; old seeds, however, are less liable to carry the organism.<sup>16</sup>

*Use of soil for inoculation of legumes.* Nodule production on plants is a result of chance contact; a large number of nodule-forming bacteria must, therefore, be present in the soil so that maximum nodule formation may take place. After the first experiments on the inoculation of legumes, it was found that certain crops, like clover, peas and beans, did not benefit from inoculation; others, like alfalfa and soybeans, could not be grown successfully without inoculation of the soil with some soil in which these crops had been grown previously. It became a common practice to spread 300 to 500 pounds of soil, taken from the upper 6 inches of a field where the particular legume had been grown successfully, over each acre of fresh soil, and disking or harrowing in before the planting of the seed. It was found<sup>17</sup> that soils once inoculated for soybeans and red clover did not need to be reinoculated when these crops were again grown in the four-year rotation. Dry soil stored for thirty months was as good for purposes of inoculation as fresh soil from the field. Further studies<sup>18</sup> have shown that there is a considerable improvement in the growth of peas in an acid silt loam, in which peas had grown eleven years previously, as a result of inoculation with artificial cultures. An acid soil may lead to a disappearance of certain nodule bacteria, the destruction of the bacteria running parallel with increasing acidity. The nodule organisms survived for fifteen years in soils which were limed, but corresponding unlimed soils showed a deficiency of the specific bacteria even when the host plant had been grown a year previously. Artificial inoculation of such a soil was found<sup>19</sup> to lead to a considerable increase in nodule formation.

The use of large quantities of soil for purposes of inoculation involves great expense and trouble in transportation and handling, aside from the introduction, with the old soil, of weed seeds and injurious microorgan-

<sup>15</sup> Feilitzen, H. v. Centrbl. Bakt. II, 23: 374-378. 1909.

<sup>16</sup> Wilson, J. K. Jour. Amer. Soc. Agron., 21: 810-814. 1929.

<sup>17</sup> Albrecht, W. A. Jour. Amer. Soc. Agron., 14: 49-51. 1922; Mo. Agr. Exp. Sta. Bul., 197. 1922.

<sup>18</sup> Whiting, A. L. Jour. Amer. Soc. Agr., 17: 474-487. 1925.

<sup>19</sup> Wilson, J. K. Jour. Amer. Soc. Agron., 18: 280-294. 1926.

isms, such as the fungi causing various wilts and nematodes. This led again to the use of pure cultures. More reliable cultures are now produced, both on artificial media and in sterile soils, as a result of the increased knowledge of the cultivation of the organisms.<sup>20</sup> In the case of certain poor sandy soils, artificial cultures can be used for alfalfa and sweet clover only if the soil has been limed well in advance of the seeding. But when no lime is used, only a heavy application of soil from an established field of alfalfa or sweet clover will give satisfactory inoculation.<sup>21</sup> Stewart still believes<sup>22</sup> that the use of soil for inoculation is in general better than seed treatment with artificial cultures.

*Commercial cultures and their preparation.* The commercial preparations of nodule bacteria commonly found on the market are in the form of liquid, agar, or soil and peat cultures. The historical process of development of the artificial culture of these bacteria is as follows: Gelatin → cotton → liquid → agar → organic → inorganic material.<sup>23</sup>

Two media are used at the United States Department of Agriculture, for the preparation of the legume cultures. One is a soil extract medium, made from 10 kgm. of field soil, 40 grams CaO and 100 liters of tap water. Ten grams of cane sugar and 0.5 gram  $K_2HPO_4$  are added for each liter of the extract. The reaction is adjusted to slight acidity to prevent the precipitation of the phosphate. The other medium is a modification of Ashby's medium for aerobic nitrogen assimilating organisms:

Saccharose.....	100 grams	NaCl.....	20.0 grams
$K_2HPO_4$ .....	20.0 grams	Calcium carbonate....	100 grams
$MgSO_4 \cdot 7H_2O$ .....	20.0 grams	Tap water.....	2000 grams
$CaSO_4 \cdot 2H_2O$ .....	10.0 grams		

The organism is grown in square bottles containing about 200 cc. of medium, which is the quantity used for one bushel of seed. The cultures are not kept for more than a month and their efficiency is tested by inoculating plants grown in sand cultures.

<sup>20</sup> The application of inoculated soil to legume seed by Arny, A. C. and McGinnis, F. W. Jour. Amer. Soc. Agron., 13: 289-303. 1921. A comparative study between the inoculating power of artificial cultures with inoculated soil has been made by v. Feilitzen, 1909 (p. 804) and Teisler, E. Centrbl. Bakt. II, 34: 50-56. 1912; Kühn, A. Ibid., 30: 548. 1911.

<sup>21</sup> Alway, F. J. and Nesom, G. H. Minn. Agr. Exp. Sta. Tech. Bul., 46. 1927.

<sup>22</sup> Stewart, G. Alfalfa growing in the United States and Canada. New York. 1926.

<sup>23</sup> Rural New Yorker, April 20, 1915.

As an agar medium, the following<sup>24</sup> may be suggested:

Mannitol.....	10.0 grams	CaCO <sub>3</sub> .....	1.0 gram
K <sub>2</sub> HPO <sub>4</sub> .....	0.5 gram	Distilled water.....	900 cc.
NaCl.....	0.2 gram	Agar.....	15.0 grams
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.2 gram	Reaction pH.....	6.8
CaSO <sub>4</sub> ·2H <sub>2</sub> O.....	0.1 gram	Sterile yeast water...	100 cc.

The liquefied agar is allowed to solidify in the form of slants on the broad side of the flat square bottles; the solidified agar is then inoculated with a few drops of a vigorous liquid culture or a suspension of a solid culture in distilled water. The bottles are incubated at 28°. Each culture is sufficient for the inoculation of one acre. For the preparation of large quantities of medium, the following method may be employed:<sup>25</sup> 175 grams of agar are dissolved in 3000 cc. of water, by placing it in the autoclave at 10 to 15 pounds pressure; 2.25 pounds hardwood ashes are boiled in 1000 cc. of water and filtered; 0.5 gram KH<sub>2</sub>PO<sub>4</sub>, 0.5 gram MgSO<sub>4</sub>, 0.5 gram NaCl, 0.25 gram CaSO<sub>4</sub>·2H<sub>2</sub>O and 6.25 grams CaCO<sub>3</sub> are placed in 1000 cc. of hot water. The three solutions are mixed and 87.5 grams saccharose and 12.5 grams mannitol are added. The medium is placed in 1.5 ounce bottles and sterilized at 10 pounds. The bottles are then inoculated with 2 cc. of a culture of the desired organism, incubated seven days, and then distributed.

The use of good fertile soil, which has been previously sterilized, for the cultivation of *Bact. radicola* was found<sup>26</sup> to give very good results both for the propagation of the organism and as a culture for distribution. The growth of the nodule bacteria on nitrogen-rich media does not destroy the infecting power of the organisms.<sup>27</sup> A sandy soil to which some decomposed organic matter is added is air dried, then placed in ten pots and sterilized for two hours at 100°C. Water is then added to the pots to bring the soil to optimum moisture and the soil inoculated with a suspension of the culture grown in Ashbys' solution. A few cc. are used for inoculating each pot. Soil cultures last much longer than agar cultures. Each pot weighing about 680 grams can be used to inoculate one bushel of seed or one acre of land. Peat cultures of the nodule bacteria are also being used quite extensively.<sup>28</sup>

To test cultures for the abundance and vitality of the specific legume bacteria, two methods are used:

1. The culture is diluted to 1:10,000 or 1:100,000, then 1 cc. of the final dilution

<sup>24</sup> Wright, W. H. Soil Sci., 20: 95-141. 1925.

<sup>25</sup> Harrison, F. C. Trans. Roy. Soc. Canada, Ser. 3, 9: 219. 1915.

<sup>26</sup> Simon, J. Mitt. Ökon. Gesell. Sachsen., 35: 1-27. 1908; Kühn, A. Centrbl. Bakt. II, 30: 548-552. 1911; Temple, J. C. Ga. Agr. Exp. Bul., 120. 1916.

<sup>27</sup> Prucha, M. J. Cornell Univ. Agr. Exp. Sta., Mem. 5. 1915.

<sup>28</sup> Earp-Thomas, G. H. Jour. Amer. Peat Soc., 15: 18-23. 1922.

is added to 9 cc. of agar medium (Temple used a medium consisting of 10 grams sucrose, 1 gram  $\text{KH}_2\text{PO}_4$ , 15 grams agar, 1000 cc. tap water, pH = about 6.5 to 7.0) and plates prepared. These are incubated for 6 to 7 days at  $25^\circ\text{C}$ .; the number of viable bacteria, as well as abundance of contaminations, can then be determined. It is frequently difficult to differentiate on the plate between *Bact. radiobacter* and *Bact. radiculicola*,<sup>29</sup> and it is impossible to determine the specificity of the organism present in the soil.

2. To identify the strain, direct inoculation tests must be employed. Either bottles with sterilized sand containing 20 per cent moisture or tall cylinders containing sterile 0.75 per cent agar media must be used. The seeds are sterilized by treatment for fifteen minutes with a solution of 0.1 per cent corrosive sublimate, 1 per cent formaldehyde or 5 per cent hypochlorite, then rinsed in sterile water and germinated on moist filter paper in a moist chamber. The sprouted seeds are then removed with sterile forceps, dipped in the inoculating material and dropped upon the substrate, in which they are expected to grow. Controls should always be employed. The formation of nodules is an index of the activity of the culture. The purity of the culture can also be tested on sterilized potato, upon which nodule bacteria do not grow (some give some growth in 4 weeks), while common contaminations and *Bact. radiobacter* produce a growth in 5 to 7 days at  $28^\circ\text{C}$ .<sup>30</sup>

*Bact. radiculicola* multiplies very rapidly in sterile soil and the growth of the organism is greatly diminished when the sterile soil is mixed with non-sterile soil, indicating that normal soil is not a very favorable medium for its development.<sup>31</sup>

The activity of poor strains can be considerably increased by repeated passage through the host plant, as shown by more abundant growth of the plant and large size of nodules.<sup>32</sup> However, in the case of good strains, repeated plant passage may decrease their efficiency.<sup>33</sup>

*Biological types of legume bacteria.* It has been pointed out above that, although so far all the bacteria capable of inoculating leguminous plants are classified under one species *Bact. radiculicola* or *Rhiz. leguminosarum*, different morphological, serological, and cultural differences are found between the forms inoculating different plants. Morphologically they are differentiated by the formation of peritrichous or monotrichous flagellation. Serologically and culturally they are differentiated into a number of groups (3 to 11), the different representatives of each group being capable of cross-inoculation. However, even one type of plant

<sup>29</sup> Joshi, N. V. India Dept. Agr. Mem. Bact. Ser., 1: 219-276. 1920.

<sup>30</sup> Löhnis and Hansen, 1921 (p. 121).

<sup>31</sup> Duggar, B. M. and Prucha, M. J. Centrbl. Bakt. II, 34: 67. 1912.

<sup>32</sup> See Stapp, C. Ztschr. angew. Bot., 11: 197-245. 1929.

<sup>33</sup> Baldwin, I. L. Jour. Bact., 17: 20-21. 1929.



may be inoculated by strains of the organism which possess certain distinct differences.

It was found<sup>34</sup> that (1) different strains of bacteria used in inoculating soybeans differ in their nitrogen-fixing efficiency; (2) different strains of bacteria used for soybean inoculation differ in their power of producing nodules on the roots of the plants, as shown by the actual count of the number and size of nodules; (3) different varieties of beans differ in their relative "susceptibility" of inoculation; (4) the efficiency of nitrogen fixation varies with the soil composition and reaction. There is no difference in the morphology of the strains, but physiologically they may be different. This raises anew the question of the value of inoculation of soil, already inoculated, with vigorous strains of the organism.

A more liberal use of artificial cultures was frequently found to give good results.<sup>35</sup> The numbers of nodules were increased as the dose was raised from 2,500 to 20,000 bacterial cells per seed.<sup>36</sup>

*Importance of legume inoculation.* The effect of inoculation upon the growth of legumes depends to a large extent upon the physical and chemical soil conditions, such as aeration, temperature, moisture, soil composition, reaction, etc.

The effect of legume inoculation was found to consist in increasing the percentage of nitrogen in the tops and roots of the plants and the percentage of ash (excluding phosphorus) in the tops.<sup>37</sup> Inoculation alone increased<sup>38</sup> the yield of clover and alfalfa on a Colby silt loam 15.6 per cent; lime and inoculation gave an increase in yield of 49.7 per cent and in nitrogen content of 52.3 per cent. The addition of phosphorus and potassium to this soil did not give any large increase in yield. However, in the case of a poor soil, inoculation and liming, as well as applications of phosphorus and potassium, gave marked increases in crop yield; inoculation alone nearly doubled the crop yield, while the use of lime, in addition to inoculation, brought about an increase in yield of 182.8 per cent. Inoculation usually increases the percentage of nitrogen in the roots. Alfalfa showed an average gain of 87.5 pounds of nitrogen on a poor soil and only 41.3 pounds on a rich soil; soybeans properly inoculated fixed about 108 pounds of nitrogen in an acid soil

<sup>34</sup> Wright, 1925 (p. 122).

<sup>35</sup> Wilson, J. K. and Leland, E. W. Jour. Amer. Soc. Agron., 21: 574-586. 1929.

<sup>36</sup> Thornton, H. G. Jour. Agr. Sci., 19: 373-381. 1929.

<sup>37</sup> Arny, A. C. and Thatcher, R. W. Jour. Amer. Soc. Agron., 7: 172-185. 1915; 9: 127-137. 1917.

<sup>38</sup> Graul, E. B. and Fred, E. B. Wis. Agr. Exp. Sta., Res. Bul., 54. 1922.

and about 129 pounds when half enough lime needed to neutralize the soil acidity was added (fig. 83).

The use of pure cultures affords a quick and easy method for introducing into the soil of the bacteria which enable the leguminous plants to obtain nitrogen from the atmosphere. Fresh inoculation of soil with specific nodule bacteria may be of direct benefit to the crop, even if the same plant has been grown previously.<sup>39</sup> This is due to the fact that the organisms present in the soil itself may not be as vigorous as freshly introduced cultures and the small expense involved by fresh inoculation may be fully compensated by the more vigorous growth of the plants.

Nobbe and Richter found that in some cases 93 to 96 per cent of the nitrogen in vetch was obtained from the atmosphere. The addition of available nitrogen to the soil brought about a decrease in the amount of nitrogen fixed. A fixation of 92 per cent of nitrogen in the alfalfa plants as a result of inoculation was recorded.<sup>40</sup> Others<sup>41</sup> obtained a fixation of 15 pounds of nitrogen for alfalfa with soil as an inoculum and 35 pounds when a commercial culture was used for inoculation. In cylinder experiments with various legumes turned under as green manures, in a rotation of corn, potatoes, oats and rye, a gain of 54 pounds of nitrogen annually over a period of seven years, as a result of inoculation, was reported.<sup>42</sup>

Clover was found to contain at maturity an average of 27 per cent of its nitrogen in the roots; 46 per cent of the total nitrogen of alfalfa was also found in the roots.<sup>43</sup> The nitrogen content of clover and especially of alfalfa inoculated with the proper bacteria is greatly increased as a result of inoculation. In some soils the increase was 171.2 per cent greater than that of the untreated control. On adding 2.5 tons of  $\text{CaCO}_3$  per acre, in addition to inoculation, the increased crop yield was 310.7 per cent more than the control. Inoculation also increased the nitrogen percentage in the roots and vines.<sup>44</sup> In case of soybeans an average increase of 100 pounds of nitrogen per acre from inoculation, in pot experiments, and 24 pounds in field experiments was recorded.

<sup>39</sup> Fred, E. B. and Bryan, O. C. *Soil Sci.*, 14: 413-415. 1922.

<sup>40</sup> Alway, F. J. et al. *Neb. Agr. Exp. Sta. 25th Ann. Rpt.*, 25: 56-65. 1912.

<sup>41</sup> Lipman, J. G. *N. J. Agr. Exp. Sta. Bul.*, 227. 1910.

<sup>42</sup> Lipman, J. G. and Blair, A. W. *Soil Sci.*, 1: 579. 1916.

<sup>43</sup> Brown, P. E. and Stallings, J. H. *Soil Sci.*, 12: 365-307. 1921.

<sup>44</sup> Graul and Fred, 1922 (p. 808); Fred, E. B. *Soil Sci.*, 11: 469-477. 1921; Whiting, A. L., Fred, E. B. and Stevens, J. W. *Wis. Agr. Exp. Sta. Bul.*, 372. 1925.

Inoculation increased the yield of soybeans 1787 pounds per acre, or more than threefold; it also resulted in a net gain of nitrogen of 57 pounds per acre, 87 per cent of which was in the tops. The residue left after the crop has been removed, also benefits the succeeding crop. An average increase in the total nitrogen content of the crop, as a result of inoculation, is given as 122 pounds per acre for American soil, and 200 pounds per acre for German soil.

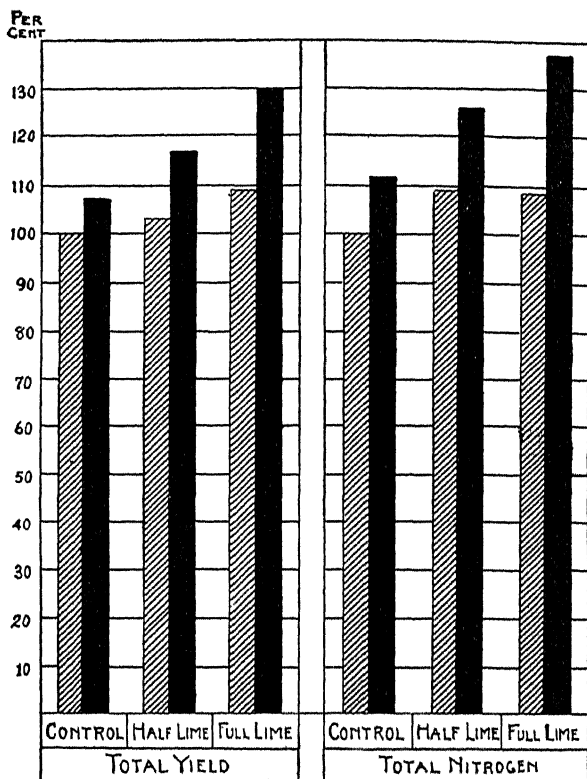


FIG. 83. Influence of inoculation and liming upon the growth and nitrogen content of alfalfa: checked columns denote uninoculated and dark columns inoculated soil (from Fred and Graul).

*Inoculation of non-leguminous plants with nodule bacteria.* Various attempts have been made to inoculate nodule bacteria upon non-leguminous plants, with variable success. Burrill and Hansen,<sup>45</sup> basing

<sup>45</sup> Burrill and Hansen, 1917 (p. 121).

their conclusions on their own observations and reports of other investigators, reported the results to be absolutely negative. Blunck,<sup>46</sup> however, reported positive results. He grew the organisms first on a synthetic medium, then added to the medium an extract of the roots of the non-leguminous plant, then he grew the organism on the sterile dead root of the plant, and finally on the living root. By this process of gradual adaptation, Blunck claims to have obtained positive results. However, in view of the fact that these results have never been confirmed, nor has Blunck himself brought further evidence to substantiate his hypothesis, we must consider the results as doubtful. Various other claims to discoveries of cultures of symbiotic nitrogen-fixing bacteria adapted to non-leguminous plants are usually found to be worthless on careful study. As far as our present information is concerned, non-legumes cannot yet be inoculated with nitrogen fixing bacteria with beneficial results.<sup>47</sup>

*Inoculation of soil with non-symbiotic nitrogen fixing bacteria.* It has long been known that a soil is capable of moderating in some way the losses of nitrogen due to removal in crop, drainage, etc. From various practical observations, it is claimed that one hectare of soil of Central Europe is capable of fixing between 10 to 60 kilograms of nitrogen per year, independent of leguminous plants.<sup>48</sup> The most abundant and most active of the non-symbiotic nitrogen fixing organisms are the species of *Azotobacter* and *Cl. pastorianum*. As pointed out elsewhere, the most important factors influencing the activity of non-symbiotic nitrogen-fixing bacteria in soil are (1) the degree of activity of the specific organisms; (2) amount of available carbohydrates and other carbon compounds in the soil, which could serve as sources of energy for nitrogen-fixation; (3) presence of mineral substances, especially calcium, phosphates, potassium, iron, etc.; (4) proper soil aeration favoring the activity of aerobic forms and not injuring the development of anaerobes (together with the aerobes); (5) presence of sufficient water; (6) proper soil temperature; and (7) favorable soil reaction, etc. These factors have been considered in detail elsewhere. It is sufficient to emphasize here that, in addition to the presence of vigorous nitrogen fixing bacteria, the soil must be in a proper physical and chemical condition, before nitrogen-fixation will take place. If soil conditions are made favor-

<sup>46</sup> Blunck, G. Centrbl. Bakt. II, 51: 87-90. 1920.

<sup>47</sup> Kordes, H. Ztschr. Pflanzen. Düng. Bodenk., 4B: 382-394. 1925.

<sup>48</sup> Omeliansky, W. L. Russian Jour. Microb., 2: 125-139. 1915.

able, the nitrogen fixing bacteria will develop rapidly, since they are present in sufficient abundance in all soils. In acid soils, no infection with *Azotobacter* is apt to occur soon after the reaction is corrected; however, after a certain interval of time these organisms will become established in the soil. It is also important to emphasize that a sufficient amount of energy is required for nitrogen fixation to take place, i.e., for every pound of nitrogen fixed by the non-symbiotic bacteria, about 100 pounds of available carbohydrate or other carbon compounds are consumed.

When natural organic substances, such as straw, various plant residues and green manures are added to the soil, they are first of all attacked by the numerous saprophytic soil microorganisms, especially the fungi, which break them down rapidly with the liberation of carbon dioxide as the final product. In assimilating the available carbon compounds, the fungi and other microorganisms require available nitrogen to build up their body proteins, about 2 to 5 parts of nitrogen for every 100 parts of carbon available. This nitrogen is obtained from the available nitrogen in the soil, to the detriment of the growing plants, unless it is also introduced into the soil, as in stable manure. The fungi and other saprophytic organisms will stop growing when the available nitrogen is exhausted. It is then when the nitrogen-fixing bacteria become active. In the presence of available carbohydrates or their derivatives, such as the various organic acids, and in the absence of available nitrogen, they may fix the nitrogen of the atmosphere. After all the available carbohydrate has been used up or transformed into unavailable forms, the fungus mycelium and bacterial cells, including those of the nitrogen-fixing bacteria, begin to be decomposed by the various soil microorganisms, especially the bacteria and actinomyces, with the result that the nitrogen is made gradually available for higher plants.

This group of phenomena brought about considerable confusion when an attempt was made to explain why the addition of available carbohydrates at first lowers crop yield,<sup>49</sup> and why favorable results are obtained one year after the application of carbohydrates, as shown in the following summary:

<sup>49</sup> Koch, A. et al. (p. 513); Krüger, W. and Schneidewind, W. *Landw. Jahrb.*, 29: 747-770. 1900; Gerlach, M. and Vogel, I. *Centrbl. Bakt.* II, 9: 817, 880. 1902; 10: 636-643. 1903; Lipman, J. G. *N. J. Agr. Exp. Sta.* 21 Ann. Rep. 1908, 144-147.

CARBOHYDRATE	CROP YIELD, DRY MATTER		TOTAL NITROGEN IN CROP	NITROGEN IN SOIL, SPRING OF 1906	NITRATE N
	Oats, 1905	Beets, 1906			
<i>per cent</i>			<i>grams</i>	<i>per cent</i>	<i>p.p.m.</i>
None	100.0	100.0	0.5914	0.093	10
Glucose, 2	32.8	186.0	0.6814	0.105	17
Sucrose, 2	33.3	179.0	0.6800	0.105	15
Sucrose, 4	37.7	283.0	1.0092	0.119	37

In certain experiments no effect has been noted as a result of application of carbohydrates and, in some cases, even a certain injury has been reported. This may be due to the variability of the method for determining the total nitrogen. The following example may be taken as an illustration: the addition of five tons of a pure carbohydrate (on a water-free basis), whether in the form of straw, hay, plant stubble, or green manure, is quite a large amount to add per acre of soil. Even assuming that such a quantity is added and that it is all utilized by the nitrogen-fixing bacteria as a source of energy (which is again doubtful), the maximum amount of nitrogen fixed under these conditions would be 0.5 part of nitrogen for every 100 parts of carbohydrate, or 50 pounds of nitrogen per acre. If the soil contains only 0.1 per cent nitrogen, this will form only about  $2\frac{1}{2}$  per cent of the nitrogen content of the soil, i.e., less than the error involved in the method for determining total nitrogen. By using 5 gm. of soil for total nitrogen determination, the difference will be only a fraction of a milligram. Those familiar with the method know how easily such an error is obtained.

The question of soil reaction has been also discussed in detail elsewhere. No introduction of *Azotobacter* will help to establish this organism in the soil and bring about increased nitrogen fixation in an acid soil with a pH less than 6.0. The addition of lime to change the pH of the soil to 6.0 and above will lead to a development of an active nitrogen fixing flora, if other conditions are favorable. Good results from inoculation may be obtained in the case of freshly drained swamps, in which *Azotobacter* would be absent.<sup>50</sup> But the reaction of the soil must first be adjusted by the use of lime.

All attempts to inoculate normal soils with *Azotobacter* and other non-symbiotic nitrogen-fixing organisms failed on repeated study.

<sup>50</sup> Stoklasa, J. Deut. landw. Presse 1908, No. 25-27; Stranak, Fr. Centrbl. Bakt. II, 25: 320-321. 1909.

From Caron's "alinit"<sup>51</sup> (1895) to Bottomley's<sup>52</sup> "bacterized peat," all attempts to exploit commercially the nitrogen-fixing capacity of *Azotobacter* and other bacteria failed.<sup>53</sup> The soil itself harbors sufficient organisms which become active when conditions and nutrients are favorable, as shown by Gainey for *Azotobacter*. Hiltner<sup>54</sup> claimed to have obtained good results from inoculation of sugar beets with bacteria; just what these bacteria do in the soil, has not been determined. The U-cultures of Kühn,<sup>55</sup> which are also used as an all-crop inoculant, have been found worthless by Barthel.<sup>56</sup>

Ehrenberg<sup>57</sup> compared soil inoculation with symbiotic and non-symbiotic nitrogen-fixing bacteria, with the following conclusions:

1. On comparing the abundance of various bacteria living in the soil, hardly any change takes place as a result of artificial inoculation, since the bacteria from the commercial preparations rapidly succumb. The legume bacteria have the opportunity of penetrating the roots of the leguminous plants, whereby they are protected from competition with other bacteria. The protection is afforded also when the leguminous plants are dead since the nodules do not decompose so readily.

2. Although soil bacteria fix appreciable quantities of nitrogen under laboratory conditions and a definite success may be obtained on inoculating soil with such bacteria, the use of expensive substances like mannitol or sugar makes it rather prohibitive. The growth of algae was found to offer only questionable hopes.<sup>58</sup>

*Soil inoculation with autotrophic bacteria.* The growth of a number of organisms causing plant diseases can be checked by a proper control of the soil reaction; this is especially true of those pathogens that are very sensitive to acidity, as in the case of diseases caused by actinomycetes

<sup>51</sup> Caron, A. Landw. Vers. Sta., **45**: 401-418. 1895; Stoklasa, J. Centrbl. Bakt. II, **4**: 39-41, 78-86, 119-130, 284-289, 507-513, 535-540. 1898; Deut. landw. Presse, **35**: 274, 286-297. 1908; Heinze, B. Centrbl. Bakt. II, **8**: 391, 417, 449, 513, 545, 609, 663. 1902.

<sup>52</sup> Bottomley, W. B. Rpt. Brit. Assn. Adv. Sci. 1911, 607-608.

<sup>53</sup> Russell, E. J. Jour. Bd. Agr. (London), **24**: 11-20. 1917.

<sup>54</sup> Hiltner, L. Mitt. deut. landw. Gesell., **26**: 243. 1921; Engelmann, E. Mitt. deut. landw. Gesell., **37**: 560. 1922.

<sup>55</sup> Kühn. Deut. landw. Presse, **44**: 467. 1917.

<sup>56</sup> Barthel, Chr. Meddl. No. 184, Centralanst. f. forsoksv. jordbruks. 1919.

<sup>57</sup> Ehrenberg, P. Fühl. landw. Ztg., **69**: 161-166. 1920; Centrbl. Bakt. II, **53**: 409. 1921.

<sup>58</sup> Further information on the inoculation of soil with *Azotobacter* is given by Emerson, P. Iowa Agr. Exp. Sta. Res. Bul. **45**, 1918; Omeliansky, 1923 (p. 495); Brown, P. E. and Hart, W. J. Jour. Amer. Soc. Agr., **17**: 456-473. 1925; Gainey, P. L. Soil Sci., **20**: 73-86. 1925; Kansas Agr. Exp. Sta. Tech. Bul. **26**. 1930.

(potato scab, sugar-beet scab). The addition of sulfur to soil is used as a means of increasing the acidity of the soil to a point at which the development of the disease-producing organism is prevented. But before this can take place, the sulfur has to be oxidized to sulfuric acid by proper bacteria. All soils contain organisms capable of oxidizing elementary sulfur; in some instances, however, these may carry on the oxidation only very slowly. When strong sulfur oxidizing organisms are added, the oxidation of the sulfur may be hastened appreciably. This led to the development of a commercial product, which consists of sulfur inoculated with a crude culture of *Thiobacillus thiooxidans*. It still remains to be proven, however, how long the culture will survive on the dry sulfur and how efficient it may be, in comparison with the organisms present in ordinary soils.

The inoculation of soil with nitrifying bacteria, especially in case of freshly drained swamps may also be of direct benefit.

*Inoculation of soil with heterotrophic, non-nitrogen-fixing microorganisms.* Attention has already been called to Caron's first attempt to prepare a bacterial culture (alinit<sup>59</sup>) for soil inoculation, with the idea of stimulating the decomposition of organic matter in the soil. Although the first attempts were unsuccessful, references are still found in recent literature concerning the use of similar preparations. It is claimed<sup>60</sup> that *B. ellenbachensis*  $\alpha$  and alinit-bacillus  $\alpha$  will allow luxuriant growth of grain crops without the addition of nitrogenous fertilizers. Various other preparations (A. Kühn's U-cultures, All-crop Inoculant, Inoculin) have been placed on the market for the inoculation of cultivated plants other than legumes. The results have so far proven negative.

It still remains to be seen whether the inoculation of soil with strong cellulose-decomposing bacteria can stimulate the processes of decomposition of organic matter in the soil.

We need also mention here again the results of Hartley<sup>61</sup> showing that soils partially sterilized by means of heat or volatile antiseptics will benefit by the inoculation with saprophytic fungi. These fungi grow rapidly in the treated soil and thus prevent development of parasitic fungi causing the damping off of forest seedlings. If a soil is infested with injurious nematodes, it may be benefited by inoculation with predatory nematodes.<sup>62</sup>

<sup>59</sup> Complete literature on alinit is given by Heinze, 1902 (p. 813).

<sup>60</sup> Daude. Blätt. Zuckerrüben., 25: 156. 1919; 26: 30, 45, 176. 1919; Centrbl. Bakt. II, 53: 408. 1921.

<sup>61</sup> Hartley, 1921 (p. 797).

<sup>62</sup> Steiner and Heinley, 1925 (p. 331).



## CHAPTER XXXIV

### HISTORY OF SOIL MICROBIOLOGY

"The history of a science is not merely a chronicle of discovery, but a study of the relation of methods and ideas in progress and the application of the conceptions thus gained to guide us in present and future work."—*Henderson*.

Every biological science goes through, in the course of its development, a series of stages which can be briefly summarized as follows:

1. Ecological stage, including description and classification.
2. Physiological stage, or a study of the activities of the organisms in question.

3. Experimental stage, whereby changes in the physiology of the organisms are studied, as a result of experimental conditions.

4. Mathematical stage, when formulae are developed to express in exact language the mechanism of the physiological processes.

Finally, we find that every science, when it reaches a certain stage of development, branches off into several new sciences.

The age of a science is definitely indicated by its stage of development. Soil microbiology is only a science in the making; but, while it has not left as yet the ecological stage and the very methods of study are still undergoing active change, it has already reached the stage when expressions are found for a correlation between the activities of the microorganisms and the environmental conditions. This science includes not only a study of the microbial population of the soil and the biochemical processes brought about under experimental conditions, but also the resultant phenomena of the sum total of the activities of this population in the soil. The fact that numerous groups of organisms, the activities of which may be supplementary or antagonistic, exist in a very complex medium, the soil, under very complex environmental conditions, tends to complicate the subject still further. The science of soil microbiology is also beginning to branch off into soil mycology, algology, protozoology, nematology, as well as soil biochemistry.

*Beginnings of soil microbiology.* Each science has its roots and antecedents in the past and each is developed out of the materials of the past. This is true particularly of soil microbiology, which has developed

directly from the empirical practices in agriculture and as a result of the advances made by the science of bacteriology; it owes a great deal to the older sciences, botany and its daughter science mycology, zoology and its offspring protozoology, chemistry, physics, and especially these sciences as applied to soil processes.

Among the empirical practices, we need mention, (1) the beneficial influence of the growth of legumes upon subsequent crops, (2) the composting of manure or various farm wastes, (3) the burning of the upper layer of soil to insure better crops, (4) the addition of fertile soil to soil newly prepared from bogs. The progress of physics resulted in the development of the microscope and balances. The progress of chemistry resulted in a knowledge of the chemical composition of matter, a better understanding of the composition of complex proteins and carbohydrates, and in the development of various methods used in organic and inorganic analysis. The development of physical chemistry resulted in the progress of our understanding of the nature of colloids and surface phenomena, of the hydrogen-ion concentration of the medium, oxidation and reduction processes. All have contributed to the development of soil microbiology. The study of the microorganisms themselves dates back to the work of Kircher and Leewvenhoek (1683), who made the first observations on the bacteria, followed by the investigations of O. G. Müller (*Animalcula infusoria*, 1786), Ehrenberg<sup>1</sup> and T. Schwann.<sup>2</sup> The last demonstrated that yeast was a living organism. The science of botany has contributed to a better knowledge of the morphology and physiology of fungi and algae. The science of zoology advanced our understanding of the protozoa, nematodes and other invertebrates found in the soil, especially in respect to their nutrition and relation to the other members of the soil population.

Bacteriology, beginning with the work of Pasteur on microorganisms as chemical agents, has been one of the most fruitful fields in stimulating the development of soil microbiology. Both medical and agricultural bacteriologists have made important contributions. It is sufficient to mention the methods of pure culture study of bacteria, finally leading to a differentiation of microorganisms on a physiological basis; the plate method for counting and isolating bacteria; the introduction of selective enrichment and specific culture media, the anaerobic methods, etc., all of which were necessary steps in the progress of the science. The de-

<sup>1</sup> Ehrenberg, G. Chr. *Die Infusionstierchen als vollkommene Organismen*. 1839.

<sup>2</sup> Schwann, T. *Gilbert's Ann. Phys. u. Chemie.*, 51. 1837.

velopment of media proceeded from the complex organic media, introduced by R. Koch, to the special inorganic media such as silica gel, introduced by Winogradsky, and synthetic media introduced by Beijerinck. These artificial media finally led to the use of the soil itself as a culture medium for the growth and activities of microorganisms. Any modification of the physical or chemical condition of this medium, either as a result of addition of nutrients or stimulants, or as a result of change of environmental conditions, leads to a change in the numbers and activities of the microorganisms.

Three distinct biological processes had been clearly outlined and partly understood by the middle of last century: (1) *Decomposition of organic matter*. This was known to give rise to humus which was believed to be one of the fundamental principles in soil fertility. Some investigators considered humus only as an intermediary product and not as a plant food; organic matter was believed to decompose slowly by chemical oxidation. The work of Schloesing, Wollny and others finally led to a better understanding of the process. (2) *Nitrification*. The accumulation of nitrates in the soil as a result of decomposition of organic matter was known in the 17th and 18th centuries, but only Bous-singault connected this process with soil fertility. (3) *Nitrogen fixation*. The use of legumes for enrichment of the soil was known to the ancient Romans. Berthelot was the first to suggest that nitrogen fixation may be accomplished also by non-symbiotic bacteria. The isolation of the organisms concerned both in nitrification and nitrogen fixation took place only at the close of last century.

From the beginnings of soil microbiology, when the rôle of microorganisms in soil processes was becoming clearly understood and properly appreciated up until the present time, four distinct periods can be clearly recognized.

Formative period—from 1860 until 1882.

Period of rapid expansion—1882 until 1902.

Period of agronomic interpretation and intensive application—1902 until 1912.

Comprehensive study and appreciation of the various members of the soil population and their complex interrelations—1912 to date.

*Soil microbiology as an independent science.* Three definite and often distinct conceptions are included in the science of soil microbiology, namely: (1) a knowledge of the organisms occurring in the soil, their numbers, types and relationships; (2) the biochemical activities of these organisms, under laboratory conditions and in pure culture; (3) the

rôle of these activities in the soil processes and their application to agriculture. Any advance in the science of botany, zoology, or bacteriology, which throws light upon the nature of organisms which occur in the soil, such as the development of new methods, a better system of classification in bacteriology, the rôle of bacteria in the nutrition of protozoa, the question of the physiology and classification of filamentous fungi, the rôle of mycorrhiza in plant nutrition, etc., can be considered as contributing to the advance of soil microbiology. Any discovery in the field of chemistry or physics, which has a bearing upon soil formation and composition as well as processes taking place in the soil, upon the chemistry of plant cells and the development of new methods of analysis, contributes to the progress of our knowledge of the microbial population of the soil, its activities and importance in soil science.

It is argued, however (Winogradsky), that, while considerable information has accumulated concerning the methods of isolation and cultivation of certain organisms present in the soil, while a great many organisms have been isolated and described, while the biochemical activities of a number of these organisms are known, there is still lacking a science of soil microbiology proper, or an applied science.

The beginnings of soil microbiology as an independent science date back to the sixth and seventh decades of the last century. There are two outstanding names in soil chemistry and bacteriology, whose theories were far from agreeing, but whose researches have dovetailed to give origin to the science under consideration; namely, those of Liebig (1840) and Pasteur (1860). Liebig's theories of soil fertility fell short because he did not recognize the activities of microorganisms. Pasteur's work was not concerned directly with soil microorganisms, but his bacteriological investigations in general, and specifically the study of the various fermentations including that of urea and butyric acid, pointed the way to a new development.

It remained for the practical agriculturist to combine the efforts of the chemist and bacteriologist and call attention to the importance of microorganisms in soil fertility. Kette<sup>3</sup> (1865) deserves the credit for being the first to recognize this fact. He advanced the fermentation theory, in which he stated that the importance of the addition of stable manure to soil was due to the fact that it cannot be replaced by inorganic nitrogen compounds and minerals or by purely vegetable matter, because these lack "a true vibron fermentation." His views have found

<sup>3</sup> Kette, W. Die Fermentationstheorie gegenüber der Humus-Mineral und Stickstofftheorie. 2 Aufl. 1865.

ardent adherents, as can be recognized from the work of Rosenberg-Lipinsky,<sup>4</sup> who stated that "milliards of lower animals per acre are born every moment and die after a few days, sometimes after a few hours, serving others as food." The birth, or rather awakening, of medical bacteriology in the early part of the ninth decade of last century was also accompanied by a series of brilliant contributions to our knowledge of the bacteria of the soil. The work of Koch on the gelatin plate method, of Hellriegel and Wilfarth on the nodule bacteria and fixation of nitrogen by leguminous plants; the work of Frank and Beijerinck on the isolation of the organisms and their cultivation in pure culture, and the work of Winogradsky on the autotrophic bacteria were the contributions which transformed soil microbiology from its preparatory into the building period.

With the introduction by Robert Koch, in 1881, of the gelatin plate for the study of bacteria, a stimulus was given to the systematic study of soil microorganisms, although the earliest investigators were medical men and were more interested in public health and hygiene than in soil processes. They limited themselves entirely to a study of the numbers of bacteria and fungi in various soil layers, that would develop on the gelatin plate. Any organism that did not develop on the plate was not considered to be of importance. The occurrence of specific bacteria was studied chiefly from the point of view of finding out whether the soil contained pathogenic organisms. Here may be mentioned, in addition to Koch, Fränkel in Germany and Houston in England.

Of the biochemical processes in the soil, the first to attract universal attention were those of nitrification and nitrogen-fixation. The formation of nitrate as a result of decomposition of animal and vegetable substance has been known for many years; the nitrate necessary for the manufacture of gunpowder during the 17th and 18th centuries was obtained from composts of decomposing organic matter; however, the exact nature of the process was not understood. Liebig<sup>5</sup> considered ammonia as the most important form of nitrogen suitable as a plant nutrient and did not attach sufficient importance to the process of nitrate formation. Way<sup>6</sup> showed in 1856 that the addition of nitrogenous fertilizers to soil results in the formation of nitrates, but he did not recognize the rôle that this process plays in plant growth and it was

<sup>4</sup> von Rosenberg-Lipinsky, A. *Der praktische Ackerbau*. 3 Aufl., 2: 27. 1869.

<sup>5</sup> Liebig, J. *Principles of agricultural chemistry with special reference to the late researches made in England*. 1855.

<sup>6</sup> Way, J. T. *Jour. Roy. Agr. Soc.*, 17: 123-162. 1856.

left to French chemists to discover the nature of the process of nitrate formation in the soil and the importance of nitrates in plant nutrition.

The rapid gain in the understanding of bacteria as chemical reagents made during the sixth and seventh decades of last century tended to bring out the possible rôle of bacteria in soil processes, in contradistinction to the purely chemical processes as conceived by Liebig. In the study of the purification of sewage water, Pasteur suggested in 1862 that nitrification is due to bacterial action. Schloesing and Müntz<sup>7</sup> found that when a stream of sewage was allowed to pass very slowly through a column of sand and limestone the ammonia in the sewage was at first unaffected, but, after 20 days, it became converted into nitrate, so that later the ammonia disappeared and only nitrate was found in its place. The addition of a little chloroform vapor stopped the process completely; when the chloroform was removed and a little soil suspension added, the process was started again. The rôle of "organized ferments" in the process of nitrification was thus established.

The application of this discovery to soil processes was made by Warington,<sup>8</sup> who found that ammonium salts in solution could be changed to nitrates by the addition of a trace of soil; chloroform and CS<sub>2</sub> stopped nitrate formation in the soil itself. Warington clearly distinguished two processes: 1. the conversion of ammonia to nitrite and 2. the oxidation of nitrite to nitrate. Unfortunately he failed to isolate the organisms concerned in these processes, due to the fact that he used for that purpose the gelatin plate method. This important problem in microbiology was finally completed by Winogradsky in 1890.

The progress made in the study of the problem of nitrogen fixation by leguminous plants can be separated into the following stages: 1. Our knowledge dating back to the time of the Romans that legumes enrich the soil. 2. The work of Lachmann (1858), Woronin (1866) and other botanists on the formation of nodules by the roots of the leguminous plants. 3. Boussingault emphasized in 1838 that the favorable action of legumes upon soil is due to their power of fixing atmospheric nitrogen; Frank demonstrated in 1879 that the nodules on the roots of the plants are formed as a result of inoculation with microorganisms. 4. The investigations of Hellriegel and Wilfarth, who have shown that, while the growth of non-leguminous plants is proportional to the amount

<sup>7</sup> Schloesing and Müntz. *Compt. Rend. Acad. Sci.*, **84**: 301-303. 1877; **85**: 1018-1020; **86**: 892-895. 1878.

<sup>8</sup> Warington, R. *Jour. Chem. Soc.*, **33**: 44-51. 1878; **35**: 429-456. 1879; **45**: 637-672. 1884; **59**: 484-529. 1891.

of nitrate added to the soil, there is no such relationship in the growth of leguminous plants. There was no gain in nitrogen when the nitrogen content of the plant was added to that of the sand in which non-leguminous plants were growing, but there was a considerable gain in combined nitrogen by the leguminous plants; Hellriegel and Wilfarth concluded, therefore, that the legumes took the nitrogen from the air through the agency of bacteria existing in the nodules of their roots. 5. Schloesing and Laurent<sup>9</sup> found that the weight of the nitrogen absorbed from the air by the leguminous plant was about equal to the gain in nitrogen by the plant and the soil. 6. The final isolation of the organism (*Bact. radicicola*) by Beijerinck.

The discovery of the process of non-symbiotic nitrogen-fixation in the soil and the agents responsible also forms one of the most fascinating chapters in the science of Soil Microbiology. Berthelot was the first to attract attention to the possible rôle of microbes in the process. In 1885, he published a note entitled "Considerations générales sur la balance de l'azote," in which he states that the natural reserves of nitrogen compounds would tend to diminish if not for the compensating factors; there must exist, therefore, agents capable of fixing nitrogen. This preconceived idea led him to investigate first the rôle of atmospheric electricity, then of soils and the microscopic organisms which it contains. At first he believed that the algae are the responsible agents, but later, as a result of a study of bacteria isolated by the gelatin plate method, he came to the conclusion that it was the chlorophyll-free microorganisms, especially the bacteria, which are active in the process. However, his methods were entirely open to criticism and his own work lacked experimental evidence. His soils were dry and very low in organic matter, conditions under which practically no bacterial action, especially fixation of nitrogen by non-symbiotic bacteria will take place. Berthelot's ideas were later confirmed by Winogradsky.

The use of the ordinary methods, at that time current among medical bacteriologists, for the cultivation and isolation of bacteria could not lead to rapid progress in the study of specific soil organisms, as in the case of the nitrogen-fixing and nitrifying bacteria.

The introduction of the principle of elective culture enabled the selection of proper media and methods for the cultivation and isolation of specific soil organisms. A medium rich in readily available carbohydrates, such as mannitol or glucose, becomes rapidly infected by a

<sup>9</sup> Schloesing and Laurent. Ann. Inst. Past., 6: 65-115. 1892.

great many organisms capable of using the carbohydrate as a source of energy, provided available nitrogen is present. In the absence of available nitrogen, the carbon source is not attacked unless organisms are present which are capable of using the gaseous atmospheric nitrogen. When specific conditions are created for the selective growth of a specific organism, only this organism would develop. Two possibilities, however, presented themselves, namely the presence of atmospheric oxygen and its absence. Two different types of bacteria were found to be active under these conditions, an aerobic form and an anaerobic form. As a result of a mere laboratory coincidence, the bacterium which is ordinarily more difficult to isolate, namely, *Clostridium pastorianum*, was first isolated and cultivated by Winogradsky, while the aerobic form, namely *Azotobacter chroococcum*, was discovered only six years later by Beijerinck. A number of other organisms capable of fixing atmospheric nitrogen under artificial laboratory conditions were since isolated, but their ability of fixing nitrogen in soil under natural conditions still remains to be established.

The very method of demonstration and isolation of *Azotobacter* from soil has a history behind it. First the use of the standard mannitol solution of Beijerinck, then the soil extract mannitol solution agar, finally the silica gel medium of Winogradsky and the direct method of enrichment of soil.<sup>10</sup>

The names of Winogradsky and Beijerinck stand for the most fundamental work that has been done in the development of the science of soil microbiology. While Winogradsky limited himself largely to the study of autotrophic and the anaerobic nitrogen-fixing organisms, Beijerinck's contributions were distributed throughout the whole field of soil microbiology. His studies embraced symbiotic and non-symbiotic nitrogen-fixing bacteria, sulfur-oxidizing bacteria, nitrate and sulfate reducing bacteria, actinomycetes, algae, etc.

The rôle of bacteria in the decomposition of organic matter and in the liberation of the nutrients for the growth of higher plants was also developed during the ninth decade of last century. The study of decomposition of nitrogenous organic compounds in the soil is closely connected with the names of Pasteur (1863), Müntz and Coudon (1893) and Marchal (1893), who pointed out that various bacteria and fungi are capable of breaking down proteins with the rapid formation of ammonia. Here belongs also the work of Gayon and Dupetit (1881)

<sup>10</sup> Winogradsky, S. *Chimie et Industrie*, 11: No. 2, 1924.



on nitrate reduction and of Dehérain (1886) on the decomposition of farmyard manure. Wollny<sup>11</sup> and Laurent<sup>12</sup> were the first to study the decomposition of organic matter as a whole by microorganisms. The beginning of the study of cellulose decomposition by bacteria is closely connected with the name of Omeliansky, but neither the organisms nor the chemistry of the process were completely understood for a long time. Attention should also be called in this connection to the important investigations of Ferdinand Cohn on the classification and description of a number of heterotrophic soil bacteria, followed by the work of A. Meyer and his associates on the spore-forming bacteria of the soil, as well as by Chester and others.

Caron's ideas (1895) concerning the beneficial effect of soil bacteria on plant growth stirred up considerable popular interest, although his evidence was open to criticism. Caron demonstrated that any soil treatment which leads to an increase in the number of microorganisms also leads to an increase in crop productivity; fallowing of a heavy soil can be used in place of green manure. Although the practical agriculturists, by pointing out the great importance of microorganisms in soil processes and, therefore, in agriculture, often aroused great interest in these processes, such interest rarely led to fundamental contributions to the subject. The practical men expected that soil microbiology would revolutionize agriculture just as medical bacteriology revolutionized medicine but this did not materialize. Where this interest was at first most pronounced, especially in Germany and in the United States, some people came to believe that, outside of legume inoculation, there is nothing to the whole science of soil microbiology. This attitude toward a science which lies at the very basis of all soil economy and will no doubt influence, in the future, the whole agricultural practice, could result only from a lack of sufficient knowledge concerning the problems under consideration.

The soil is a three-phasic medium, rich in colloidal constituents and containing a great mass of microscopic forms of life. These produce in the soil various physical and chemical changes which are of greatest importance to the growth of higher plants. The pathologist can study the action of his organisms *in vivo*; the microbiologist working on fermentation processes can sterilize his medium, without altering its composition greatly, and inoculate it with a pure culture of the organism concerned; the soil microbiologist, however, has great difficulties in

<sup>11</sup> Wollny, E. *Bied. Centrbl. Agr. Chem.*, **13**: 796-814. 1884.

<sup>12</sup> Laurent, E. *Bull. Acad. Roy. Belg.*, **2**(3): 128-143. 1886.

attempting to learn just what the particular organism does in the soil. When the soil is sterilized, it is no longer, biologically and chemically, a normal soil. In a pure culture, free from stimulating and competing influences of other microorganisms, an organism may manifest certain activities which would not take place in the soil, or *vice versa*. It is even possible that, in pure culture, races different from those present originally in the soil develop and it is quite probable that the biochemical action is often quite different. As a matter of fact, numerous soil organisms develop upon artificial media only with great difficulty and are often repressed there by certain types which may be only occasional visitors in the soil.

*Recent advances of soil microbiology.* During the first decade of the present century, the methods used in the study of soil biological processes have undergone various modifications. Some investigators centered their attention upon the study of the metabolism of specific soil microorganisms, especially the mechanism of transformation of organic or inorganic substances as bearing upon soil processes. This was determined either by adding a small amount of soil to a sterile solution containing the specific substance, then measuring the change that took place after a definite period of incubation; or by adding the specific substance to the soil, keeping it at optimum moisture and temperature for a definite length of time, and then measuring the change. In most of these studies, the organisms responsible for the change were not considered at all. In the study of protein decomposition, ammonia was usually taken as an index, without considering the fact that the process can be carried on by numerous types of organisms and various associations and combinations, each resulting in a different amount of ammonia accumulating. In the study of nitrogen fixation, the fact was usually left out of consideration that different bacteria are active at different reactions and, therefore, different amounts of nitrogen are fixed under laboratory conditions, which may or may not hold true in the field. In the study of nitrification, the fact that the addition of large amounts of ammonium salts will soon result in a reaction (the degree depending on the buffer content of the soil) injurious to nitrification, while the addition of a considerable quantity of organic nitrogenous material may result in the formation of such large amounts of ammonia that the nitrifying bacteria will be injured, were usually left out of consideration. These investigators, often referred to as the physiological group, consisted of practical men primarily interested in the phenomena resulting from soil processes brought about by the or-

ganisms active in the soil, rather than in the organisms themselves. The first representatives of this group are Remy and Löhnis, later followed also by various workers in Germany, J. G. Lipman and Brown, Stevens and Withers, and others in America, with Perotti in Italy, Christensen in Denmark, and others contributing more to one or another phase of the subject.

The other group of investigators, often referred to as the botanical group, were more interested in knowing how many bacteria there are in the soil, what these bacteria are, and if physiological groups were studied, they wanted to know the numerical relation of one group to another. Hiltner and Störmer (1902) were the strongest advocates of this method of attack, followed by H. Fischer in Germany, Chester, H. J. Conn and others in this country, etc. In addition to these two groups of investigators interested in soil biological processes chiefly from the standpoint of the soil, a number of botanists, zoologists, general microbiologists, and chemists continued to make definite contributions to the science of soil microbiology, either by the study of one more group of soil organisms, including the soil bacteria (A. Meyer and associates, Ford et al.), fungi (Hagem, Lendner, Dale, Abbot, etc.), algae (Chodat, Esmarch, Bristol,) actinomyces (Krainsky, Conn, Drechsler), protozoa (Wolff, Goodey, Cutler, Sandon) and invertebrate animals (Cobb, Micoletzky, Steiner), or by the study of one chemical process in the soil and the organisms concerned, such as cellulose decomposition (Omeliansky, Barthel, Pringsheim, Kellermann et al.), nitrogen fixation (Bredemann, J. G. Lipman, Christensen, Gainey, Winogradsky), evolution of  $\text{CO}_2$  (J. Russell, Stoklasa, van Suchtelen, Neller).

The more outstanding recent contributions to the science of soil microbiology deal with microorganisms non-bacterial in nature. It is sufficient to mention the work of Russell and his associates on the occurrence of protozoa in the soil and on the phenomenon of partial sterilization; the occurrence and activities of algae, fungi, actinomyces and nematodes in the soil. It is also important to call attention to the development of methods for the direct examination of microorganisms in the soil by H. J. Conn and Winogradsky. We possess now also a better understanding of the organisms concerned in the oxidation of sulfur in the soil, while the rôle of microorganisms in the decomposition of cellulose and other polysaccharides (Hutchinson and Clayton, Khouvine, Winogradsky) has been made clearer; the problem of decomposition of organic matter as a whole in soil and the rôle of microorganisms

in the formation of soil "humus" have been also considerably advanced. A knowledge of the controlling influence of soil reaction upon the distribution and activities of soil microorganisms has influenced certain practices; the same is true of our increased knowledge of legume cultivation and inoculation (Hiltner, Whiting, Fred), of the use of green cover crops, fallowing and soil cultivation.

Winogradsky<sup>13</sup> considers, however, that what is called at the present time "soil microbiology" is nothing but a chapter of general microbiology treating of microorganisms isolated from the soil and hypothetically admitted as taking part in some processes which are characteristic of the soil. He considers the information available at the present time merely as an introduction to soil microbiology, but not soil microbiology itself. Insufficient attention is believed to be paid to the study of the biological agents responsible for soil processes, such as they take place in nature, in the original soil and under the specific soil conditions. General microbiology is based upon the obligatory pure culture method and upon the reactions carried out by these cultures under various conditions. In view of the fact that a specific organism has to compete in a certain process in the soil with numerous other organisms some of which are much more active and more specialized, and the mere ability of a given organism to carry out a certain function under laboratory conditions and in pure culture are no proof that the same will be carried out in the soil. Stress should be laid on the crude cultures of an elective character, arranged in a manner as to allow the observation of the biological activities in the soil itself.

The idea underlying the application of the so-called "direct method" consists in keeping conditions as natural as possible, without the use of isolations, pure cultures, bacteriological media, etc. An attempt is made to study the multiplication and activity of soil organisms in the original soil, by stimulating the development of a specific flora or spontaneous cultures as a result of addition of various substances or by physical means. These are then measured either microscopically or by special methods. For example, the addition of small amounts of certain sources of energy will stimulate the development of large cocci (*Azotobacter*) under aerobic conditions and of *Clostridium* under anaerobic. When available nitrogen is introduced, in addition to the carbonaceous material, numerous bacilli will appear, inhibiting the development of the cocci, due to the greater rate of multiplication of the bacilli. By

<sup>13</sup> Winogradsky, S. *Soil Sci.*, 25: 37-43. 1928.

these and supplementary methods of analysis, the soils can be divided into several types of various degrees of activity. Specific natural agents must, therefore, be distinguished from mere laboratory forms used for studying certain phenomena in pure cultures only.

*Present outstanding problems in soil microbiology.* The science of soil microbiology is in its mere infancy. New contributions open up broader and broader vistas, rich in reward both to the investigator and to practical men. The soil is the basis of all agricultural practice. The population of the soil makes the soil what it is and not a mass of debris containing all the elements necessary for plant growth in an unavailable form; sooner or later a study of this population will be recognized to be of the greatest importance in the future advance of agriculture.

We possess at the present time considerable information concerning the organisms inhabiting the soil and the chemical activities of many of these organisms, under controlled laboratory conditions; but little is known of the processes carried on in the soil itself, by the numerous representatives of the soil flora and fauna. The transformation of organic matter, the availability of the mineral elements, the fixation and transformation of nitrogen, the best means for the preservation of the nitrogen already present in the soil or manure, these are a few of the processes which depend largely upon the activities of microorganisms and which control the growth of cultivated plants. Some of the outstanding problems in the science may be suggested here:

1. Microscopic and cultural methods in soil microbiology, especially those which tend to indicate the organisms active in the soil under field conditions and their rôle in the transformations taking place in the soil.

2. The soil population, nature, extent and activities; the complexity of the population with its various associative and antagonistic influences. It is especially desirable to know what rôle the animal population, such as protozoa and nematodes, play in soil processes and how they influence bacterial activities, also the interrelation between the fungi and the bacteria and the rôle of actinomyces in the soil.

3. Transformation of organic matter in the soil, including the chemical processes involved and organisms concerned, as well as the rôle of these transformations in soil fertility.

4. The energy balance in the soil and the balance between the different soil constituents, especially the carbon and nitrogen.

5. A better understanding of the rôle of cultivated higher plants in soil transformations and the influence that they exert upon the activities of soil microorganisms.

6. Methods of modifying the soil population and its activities and a better understanding of the processes of partial sterilization, application of lime and fertilizing materials, soil inoculation, and fallowing of soil.

7. The relation between the physical, chemical, and physico-chemical conditions of the soil, such as reaction, buffer content, moisture holding capacity, temperature, and the occurrence and activities of soil micro-organisms.

These as well as a host of other problems to which attention has been called in the previous pages will not only throw light upon the different phases of soil microbiology, little understood at the present time, but will place the science where it should be, namely in the front rank of agricultural sciences.

Depending as he does upon the contributions of the protozoologist, mycologist, bacteriologist, nematologist, etc., for a better understanding of the organisms inhabiting the soil and their activities, the soil microbiologist is in a position to correlate the sum total of the knowledge gained from these investigations and throw light upon the chemical processes in the soil. The soil physicist and the soil chemist do and will contribute definitely to the understanding of the nature of the medium in which these organisms act and of the soil solution which receives the waste products of their activities and which supplies nutrients to the plants and frequently to the microorganisms. It is to the development of the science of soil microbiology as much as to any other science that we must look for the proper understanding of the soil in its ability to supply the nutrients necessary for the growth of higher plants.<sup>14</sup>

<sup>14</sup> Further information on the history and development of soil microbiology is found in the following papers and books: Fischer, 1909 (p. 695); Löhnis, 1910 (p. XIV); Löhnis, F. *Centrbl. Bakt.* II, 54: 273-307. 1921; Winogradsky, S. *Chimie et Industrie.*, 11: No. 2. 1924; 1st Intern. Congr. Microb. Paris. 1930; Waksman, S. A. *Soil Sci.*, 19: 201-249. 1925; *Proc. 2d. Intern. Congr. Soil Sci. Moscow. Comm. III*, 72-87. 1930; *Der gegenwärtige Stand der Bodenmikrobiologie.* Urban and Schwarzenberg. Berlin. 1930.



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